EXPERIMENTAL IMMUNE FEVER:
AN IN VITRO STUDY

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An *In Vitro* Study

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DEDICATION

Dedicated to Prentiss, Patricia and Karen Markle for unfailing love and support in all things.

INTRODUCTION

Fever is one of the commonest manifestations of immunity and hypersensitivity. Although many aspects of hypersensitivity reactions have been exhaustively studied, the mechanisms by which immune processes produce fever, and the relationship of fever to immune phenomena have been the subject of only few investigations. This research project was undertaken to investigate the possibility that pyrogen production by mononuclear blood cells could result from interaction of cells with antigen-antibody complexes. Mononuclear cells from peripheral blood of human volunteers were chosen for this study, since methods are available for isolation of these cells, and the monocyte is known to be a pyrogen-producing cell (1). In addition, monocytes are the probable precursors of most tissue macrophages, cells which are known to interact with antibody and antigen-antibody complexes, both *in vivo* and *in vitro*, and which may play an important role in immune fever. No previous *in vitro* studies have demonstrated pyrogen release from cells in response to antigen-antibody complexes.

HISTORICAL BACKGROUND

Endogenous Pyrogen

Fever as a manifestation of disease has been well known for centuries, yet it is only in recent decades that the pathogenesis of this response has in part been elucidated. Although most commonly associated with diseases of infectious origin, it is also a manifestation of many diverse processes

associated with inflammatory states including neoplasia, hypersensitivity reactions, infarction and mechanical injury. Dickensen (2) recognized in 1785 that fever seemed to be brought about by the absorption of the products of the injured cells of a febrile animal. However, the demonstration that fever is actually caused by factors derived from host cells was first accomplished when differentiation of "endogenous" or cell pyrogens could clearly be made from the endotoxins of gram negative bacteria.

In 1948, Beeson (3) showed that cells, predominantly polymorphonuclear leukocytes, obtained from sterile, saline induced, peritoneal exudates, were capable of producing fever when injected into a normal rabbit. He showed, moreover, that a soluble fraction of such cells obtained by mechanical lysis was the responsible factor; cell debris was not pyrogenic. Earlier work by Billroth (4) in the 19th century and by Menkin (5) in 1943 had suggested that cells from purulent exudates released a soluble pyrogen, but these preparations were very likely contaminated with bacterial endotoxin, so the observed febrile responses were of indeterminate etiology (6). However, using techniques to avoid endotoxin contamination, Bennett and Beeson (6) surveyed extracts of normal rabbit tissues for pyrogenicity and found that only peritoneal exudate cells and cells from Arthus and Schwartzman lesions were capable of producing febrile responses when injected into normal animals. This leukocyte pyrogen was shown to produce

fevers with a latent period of 10 to 15 minutes, and a peak response within one hour. The substance was heat labile and nondialyzable (7). Subsequent work has shown that the endogenous pyrogen molecule contains essential peptide bonds, has a molecular weight of 10,000 to 20,000 and requires free SH groups for activity (8,9,10). Of great significance were the findings that febrile tolerance did not appear after repeated injections and that there was no cross tolerance in animals which were tolerant to the effects of bacterial pyrogens (11). The first evidence that such an endogenously produced mediator was present in the circulation of febrile animals and that it could clearly be distinguished from the bacterial pyrogen producing the fever was presented by Atkins and Wood (12). They showed that, after injection of typhoid vaccine into rabbits, two different pyrogenic substances could be demonstrated in their serum. A pyrogen which was present in the serum gathered 5 minutes after typhoid injection, was inactive when injected into rabbits previously rendered tolerant to the vaccine. A pyrogen present in serum drawn at 2 hours, however, when the animal was febrile, was active in both normal and tolerant animals. The early appearing pyrogen produced responses similar to those induced by bacterial pyrogens; the responses of the late appearing pyrogen was similar in character to that of leukocyte pyrogen. Furthermore, when tolerance to the typhoid vaccine in the recipient animals was reversed, febrile responsive-

ness to the exogenous pyrogen returned and could be correlated with the reappearance of endogenous pyrogen in the serum of the challenged animal. So it appeared that exogenous bacterial pyrogens produced fever indirectly by liberating from the cells of the host an 'endogenous pyrogen' which effected changes in the thermoregulation of the host. Endogenous pyrogen was further differentiated from gram negative endotoxins on the basis of its full effects on animals rendered granulocytopenic and therefore relatively unresponsive to bacterial pyrogen infections (13). The endogenous pyrogen also was shown to have a potentiated effect when infused into the carotid artery, whereas intravenous or intra-carotid infusion of bacterial pyrogen produced equivalent responses. Work by Cooper (14) had shown that integrated thermoregulatory changes were mediated by the action of endogenous pyrogen in the anterior hypothalamus; the carotid infusion of endogenous pyrogen therefore presented very high concentrations at its site of action.

Endogenous pyrogen release from stimulated cells has been shown to be associated with the fever due to numerous other infectious agents. Gram positive bacteria appear to stimulate cells by a variety of mechanisms, including cellular actions of liberated toxins, phagocytosis of bacterial particles, and immune reactions. A variety of virus-induced fevers are associated with endogenous pyrogen release (15,16). Culture filtrates of various pathogenic

fungi (17), and a protein extract of cryptococci (18) appear to cause fever by inducing endogenous pyrogen production by host leukocytes, probably as a result of immune processes.

Certain non-microbial substances have been shown to be pyrogenic. For example, some prostaglandins cause fever when applied directly to the hypothalamus (19); perhaps they function normally as intermediates in responses mediated by endogenous pyrogen. Steroids such as etiocholanolone primarily act by inducing endogenous pyrogen release from cells (20).

Endogenous pyrogen from cells of different species are variously pyrogenic in other species. While rabbit endogenous pyrogen is inactive in dogs, guinea pig and mouse cell pyrogens are active in rabbits (21). Of great experimental usefulness is the activity of human endogenous pyrogen in rabbits since this provides an easy and reliable assay of human pyrogen (22).

Mononuclear Cells as Pyrogen Producing Cells

In early work by Bennett and Beeson (6,7) and by King and Wood (23), endogenous pyrogen was obtained from sterile exudates composed mainly of granulocytes. However, it was shown by Snell and Atkins (24) that small amounts of substance with activity indistinguishable from that of granulocyte endogenous pyrogen could be extracted from homogenized, uninfected rabbit tissues such as muscle, lung, heart, spleen, liver and kidney. Neither endotoxin nor sequestered polymorphonuclear leukocytes could account for the pyrogen

thus extracted.

The first direct evidence that mononuclear cells could be a source of endogenous pyrogen production come from the work of Bodel et al (25). First, it was shown that blood from patients with agranulocytosis was capable of producing endogenous pyrogen when stimulated with heat killed Staphylococcus albus. Such blood preparations had 4 percent or less neutrophils by differential count. Incubation with staphylococci of numbers of polymorphonuclear leukocytes comparable to those in these blood preparations produced only minimal amounts of pyrogen. Similarly, blood from patients with monocytic and myelomonocytic leukemia was capable of producing endogenous pyrogen when incubated with staphylococci. The numbers of contaminating granulocytes were inadequate to account for the amount of pyrogen produced. Also, normal blood monocytes, isolated by their low density, through albumin density centrifugation, and by their property of sticking to glass surfaces, according to the observations of Bennett and Cohn (26), were found to produce endogenous pyrogen in response to phagocytosis of staphylococci. Again, contaminating granulocytes could not account for the observed activity.

Hahn et al (27) showed that peritoneal macrophages harvested from oil induced exudates in rabbits also could be a source of endogenous pyrogen. Pyrogen production by these cells could be distinguished from that of granulocytes by the macrophages' capacity to produce endogenous pyrogen

for periods of time significantly longer than was possible for granulocytes under the same incubation conditions.

Further direct evidence linking monocytes and monocyte derived tissue macrophages was gathered by Atkins, Bodel and Francis (28) using tissues from rabbits previously sensitized to BCG (Bacillus Calmette-Guerin). Blood and lung macrophages when incubated with Old Tuberculin (O.T.) released amounts of pyrogen in proportion to the dose of O.T. present in the incubation mixture whereas spleen and lymph node preparations were entirely inactive. It was shown that more lung macrophages were required to produce an amount of pyrogen equal to that produced by a given number of blood leukocytes. They showed further that the lung macrophages were capable of continued endogenous pyrogen release even after the stimulus had been removed. These cells released little or no pyrogen after five hours incubation, minimal amounts after eighteen hours, and greater amounts in a supportive medium (buffer plus glucose) than in just saline, a requirement not shown for granulocytes. However, it was observed that, like granulocytes the lung cells required incubation at 37°C and their pyrogen production was inhibited by blocking protein synthesis with puromycin. That different activators may react with different tissues was demonstrated by the finding that spleen and lymph node preparations, while not responding to O.T., were capable of pyrogen release when stimulated by phagocytosis of heat-killed staphylococcus. In all of these studies it

was shown that the numbers of contaminating granulocytes were inadequate to account for the observed pyrogen release.

Work by Dinarello et al (29) showed that a preparation of liver cells was active in pyrogen production in response to stimulation by O.T., heat killed staph and endotoxin. A separation of these cells into fractions of Kupfer cells and hepatocytes and subsequent stimulation showed that the Kupfer cells were uniformly responsible for the observed activity.

Human peripheral blood monocytes isolated according to the methods of Böyum (30) and incubated in tissue culture, have been shown to release pyrogen in response to endotoxin to heat killed staph and to tuberculin (Bodel, unpublished observations). This preparation of mononuclear cells represents a cell population remarkably free of contaminating polymorphonuclear leukocytes (less than 1%) and rendered moderately free of lymphocytes by allowing the monos to fix to the plastic surface of the tissue culture flask and then decanting the remaining medium containing the lymphocytes. This is the monocyte system used in the experiments reported below. In no system has the lymphocyte, when free of other known pyrogen producing cells, been observed to release any pyrogenic substance (31).

Moreover, the monocyte is a much more potent source of endogenous pyrogen than is the granulocyte. Unlike the lower potency of lung macrophages, peripheral monocytes have been shown to produce several fold greater amounts

of pyrogen per given number of cells than do polymorphonuclear leukocytes when stimulated by etiocholanolone or when stimulated by phagocytosis of heat killed staphylococci (32). And, to date, no discernible differences had been noted in the pyrogenic response curve from a single injection nor in the shape of the dose response curve for monocytes as compared to polymorphonuclear leukocytes.

It must be noted that monocytes placed in tissue culture undergo profound morphological and metabolic changes, transforming into macrophage-like cells, epithelioid cells and multinucleate giant cells (33,34,35). However, the time required for isolation of these cells and fixation to the tissue culture flasks is approximately 6 hours and while some transformation has been observed in such cells over such a time period, they are minimal. They include an increase in cytoplasmic pseudopodia, an increase in smooth endoplasmic reticulum, more numerous lysosomes, and the appearance of cytoplasmic filaments (35). However, all of these changes are minimal, especially compared with the marked morphological changes occurring at times of 24 hours or more. Similarly, the most marked metabolic changes of the cells characteristically occur after 24 or 48 hours (34). So the pyrogen producing mononuclear cells in the present experiments are probably more like the peripheral blood monocyte than a well differentiated tissue macrophage.

Interactions between Mononuclear Cells and Antigen-Antibody Complexes

The first direct evidence that cells interact specifically with antibody in such a way as to mediate the uptake of foreign antigen by mononuclear cells came from the work of Boyden and Sorkin (36) using a rabbit spleen cell preparation. When these cells were treated with specific antisera to human serum albumin and then washed, they were capable of specifically adsorbing the antigen. Boyden (37) later demonstrated that peritoneal macrophages were the active receptor cells in such reactions, unlike polymorphonuclear leukocytes or lymphocytes. Also he demonstrated that the reaction could occur with large 'particulate' antigens such as red blood cells. When serum from guinea pigs immunized with sheep red blood cells was mixed with guinea pig peritoneal macrophages, and sheep red cells were added, the red cells were specifically adsorbed and formed "rosettes" about the macrophages.

That the interaction is specifically mediated by antibody is clear, since without specifically immune serum present there is no rosette formation (36), and there is negligible uptake of smaller antigens (38,39). Berken (40) showed that rosette formation with peritoneal macrophages occurred to a greater extent with cells from non-immune than from immune animals. And LoBuglio et al (41) noted sphering of D positive red cells, sensitized with anti-D antibodies, about leukocytes from either D positive or D

negative donors. Furthermore, Huber (42) had shown that red cells coated non-immunologically with immunoglobulins would form rosettes, and Arend and Mannik (39) showed that heat aggregated immunoglobulin could be bound to macrophages. So the reaction is apparently mediated by antibodies.

Berkin (40) demonstrated such a "cytophilic antibody" to E. coli polysaccharide. Huber et al (43) found similar results using isolated peripheral monocytes, hepatic macrophages, and a splenic macrophage preparation. However, he too observed that lymphocytes were not capable of such activity, even when stimulated with a variety of phytomitogens. However, subsequent data indicates that platelets (44), polymorphonuclear leukocytes (44), and B lymphocytes (45) have receptors for components of antigen-antibody complexes or complexes of antigen, antibody, and complement. Specific antibody-mediated uptake of antigen by macrophages has been studied in several systems. Antigens include horseradish peroxidase (38), heterologous protein antigens such as human serum albumin (39), and small polyvalent haptens (46).

Only certain classes of immunoglobulins appear to function in mediating uptake. Berkin et al (40) noted that 'cytophilic' antibody for macrophages appeared at approximately the eighth day of immunization and further noted that it had a 7S sedimentation coefficient. He also noted that the active antibodies migrated along with other slow

migrating, complement fixing antibodies. Rabinovitch (47) showed that globulins with a high sedimentation coefficient were inactive, unlike 7S fractions. In addition, treatment of the 7S antibodies with pepsin markedly diminished their binding properties. Similar results had been shown previously by Berkin et al (40). This finding implicates the Fc region of the antibody in the attachment reaction. Huber et al (42) showed that for peripheral blood mononuclear cells, IgM antibody failed to induce rosette formation, whereas IgG antibody was effective. Using purified myeloma antibodies of specific subclass, he showed that IgG_1, and IgG_3 were most effective, while IgG_2 and IgG_4 were least active. He showed further that pepsin digestion of the antibody partially reduced its activity, whereas papain digestion eliminated all binding activity. These findings were confirmed by Abramson et al (48) and LoBuglio et al (41).

In several models, both rosette formation and uptake of soluble antigens was easily inhibited by free antibody present in the reaction medium, in amounts equal to or less than those found normally in the plasma. For this reason, the relevance of the interactions observed in vitro between cells and antigen-antibody complexes to in vivo events is not clear. However, other mechanisms seemed to play a role in producing the observed phenomena. Huber et al (42) proposed that interaction between cells and complexes proceeded in two steps, first binding and then phagocytosis. They noted, as had Berkin et al (40) that in order to

observe rosette formation, the reaction had to be carried out at room temperature. At 37°C the red cells were readily phagocytosed. And it became apparent that both binding and phagocytosis were in some way intimately tied to activity of the complement system.

The role of complement in the attachment of complexes to cells has received much attention. Berkin et al (40) noted that rosette formation was not altered when the serum had been heated to 56°C, and Huber et al (42) showed that the reaction occurred in the absence of complement. However, in another series of experiments, using red cells coated with antibody alone or with antibody and complement, these authors observed that while binding and ingestion could occur in the absence of complement, the inhibitory effects of free IgG on binding were not observed when complement was present. They found that C_3 was the active complement component in this reaction, since cells coated only with C_1, C_{14} or C_{142} were not bound in the presence of free IgG, and addition of C_{567} also did not enhance the interaction. Since free IgG did not inhibit binding if added after red cell attachment had taken place, it was concluded that the function of complement was to enhance the attachment, not the ingestion step. The authors further concluded that there was a separate complement "receptor" on the monocyte. Work by Mantovani et al (49) further confirmed such a role for complement, showing, at least at low con-

centrations of red cell bound IgG, that the effect of C_3 was greater on the attachment phase than on the ingestion phase. They also noted that by adding anti-IgG to a system with no complement, ingestion of antibody coated erythrocytes was reduced more than attachment. They concluded that the role for the complement 'receptor' was primarily for attachment and that the role for the IgG receptor was primarily for ingestion. However, Abramson et al (48), using cells coated with antibodies, and coated with C_3 but then washed free of antibody, observed that neither binding nor phagocytosis took place. Thus, no direct evidence for the existence of a separate, functional complement 'receptor' was demonstrated. Furthermore, incomplete removal of antibodies from such complement coated cells resulted in some reosette formation. Nonetheless, complement appears to enhance interactions of antibody-coated particles and cells. It may act by altering the cell surface through its enzymatic activity (50,51), since pretreatment of monocytes with various proteolytic enzymes has been demonstrated to enhance both rosette formation (52) and uptake of soluble complexes (39).

The importance of complement is not so clear, however, in experiments where adherence of soluble immune complexes has been studied. Arend and Mannik (75) noted that uptake of soluble, circulating complexes of HSA and rabbit anti-HSA by the liver occurred at the same rate in normal and in complement-depleted animals. In addition, complement

did not reverse the inhibiting effect of free IgG on the macrophage-antigen-antibody complex interactions *in vitro*. IgG in buffer inhibited the interaction as well as did IgG in serum. Complement-depleted serum containing free IgG inhibited adherence just as well as did serum containing complement. The authors postulate that the role of complement in mediating interactions between mononuclear cells and soluble antigen-antibody complexes may be to potentiate phagocytosis, not adherence, perhaps by means of its peptidase activity. A number of factors have been shown to govern the uptake of non-particulate antigen-antibody complexes by mononuclear cells. The size and structural complexity of the complexes, the total numbers of antibody-receptor interactions, and allosteric effects on the Fc region induced by the antibody's interaction with antigen all may have a role in determining the extent of binding of complexes to cells.

Multiple antibody-receptor interactions are necessary for effective cellular uptake of soluble complexes. Phillips-Quagliata *et al* (46), in studies using monovalent, divalent, and polyvalent small haptens, measured the amount of binding of antibody to macrophages, and the effects of each kind of antigen on that binding. They found that there was a great increase in binding of antibody to cells when the antibody and cells were reacted in the presence of polyvalent antigen in amounts comparable to an equivalence precipitation. There was a smaller increase in bound antibody when divalent

antigen was added, and there was no increase when monovalent antigen was present over a wide range of antigen concentrations. Furthermore, large excesses of mono- or divalent hapten blocked the uptake of antibody mediated by polyvalent antigen. They noted that binding was optimal in slight antibody excess with the polyvalent antigen. In extreme antigen excess, even with the polyvalent antigen, there was no uptake of antibody by the cells over the baseline level. Noting that free IgG almost completely inhibited binding, meaning that it already has an exposed site for binding with the receptor, the authors propose that it is primarily a summation of numerous antibody-receptor bindings which accounts for the specific attachment of structurally complex antigen-antibody complexes, not allosteric effects of antigen on the antibody mediating a tighter binding between the receptor and the antibody's Fc region.

Arend and Mannik (39), however, by comparing the greater binding of soluble antigen-antibody complexes with that of aggregated IgG, concluded that efficiency of binding was not solely mediated by summation of individual binding sites. However, they also showed that large complexes of greater than 11S sedimentation coefficient selectively adhered to the macrophages. Soluble complexes of 11S or less did not adhere as well, and these smaller complexes were determined to be a molecule of antigen with one or two molecules of antibody. The complexes which bound best were,

therefore, complexes containing at least one antigen molecule and at least two antibodies. This would seem to support Phillip-Quagliata's data showing that summation of binding sites interactions does play a role, but also shows that conformational changes in the Fc region mediated by interaction with antigen may play a role in formation of a firm attachment between antibody and monocyte receptor, especially since large protein antigens produce greater conformational changes than small molecules (53). So it seems apparent then that interactions between antigen-antibody complexes and cells is not a simple one and may be a multiphase process which depends on various factors or combinations of factors such as cell type, size and structure of antigen, size and antigen-antibody ratio of immune complexes, complement mediated mechanisms, total number of interactions between antibody and receptor, and allosteric effects due to the antigen-antibody interaction itself.

Experimental Immune Fever

It has been suspected since early in this century that mechanisms of hypersensitivity could be responsible for fevers. The fever associated with known 'toxic' effects of microbes and their products, notably the endotoxin of Gram negative bacteria, were well known. However, it was postulated early in this century that the fever of certain infectious diseases might in fact be due to mechanisms of hypersensitivity (54).

In 1964, Farr was first able to produce fevers using non-bacterial heterologous protein antigens injected into

specifically sensitized animals (55). He found after giving rabbits injections of 10 mg of bovine serum albumin (BSA) three times a week for 3 to 6 weeks, that approximately 70 percent of such animals would respond with fever when given a 2 mg injection of the BSA. Numerous other antigens have proven to be pyrogenic when injected, including human serum albumin (HSA) (56), bovine gamma globulin (BGG) (57), and culture filtrates of staphylococcus (58), certain fungi, a protein extract of cryptococcus (18), Old Tuberculin (59), drugs such as Penicillin (60), and numerous other drugs (61). Febrile reactivity to some substances appears to be due to immediate humoral hypersensitivity, e.g. BSA (55) and HSA (56) and Penicillin (60), while others appear to be related to "delayed" hypersensitivity, e.g. staph (58); fever to tuberculin may be the result of a mixed response to a variety of antigen substances in the tuberculin preparation (62).

That immune fever could be produced in states of "delayed" type hypersensitivity had been clearly demonstrated when Uhr (63) and coworkers observed febrile responses in guinea pigs which had been sensitized with antigen-antibody complexes in antibody excess such that no circulating antibody was present, but in which the typical skin reactivity of delayed hypersensitivity was present. In the process of "desensitizing" such animals with a single large dose of antigen, Uhr observed that despite the loss of local skin reactivity after the infection, there was a systemic response

which included fever, lymphopenia and a later secondary antibody response (64).

It is of some minor interest that an alternative to an endogenous pyrogen mediated pathway, in the febrile response associated with delayed hypersensitivity states was proposed by Johanovsky (65). He claimed to be able to obtain by incubation of antigens such as tuberculin with mononuclear cells and cell extracts of hypersensitive animals the release of a pyrogen with response characteristics unlike that of endogenous pyrogen. It was a substance which could act with no latent period, which could, unlike endogenous pyrogen, be produced at 4°C and could be produced in the absence of intact cells. Work at Atkins and Heijn (66), however, did not corroborate these findings; rather it was shown that BCG (Bacillus Calmette-Guerin)-sensitized cells incubated in the presence of tuberculin released endogenous pyrogen. In addition, normal, i.e. non-sensitized, blood cells incubated in the plasma from sensitized animals would also release endogenous pyrogen when stimulated by tuberculin in vitro. This implies that the fevers produced by tuberculin may well have had an antigen-antibody mediated component, since mixtures of tuberculin with sensitized rabbit plasma in vitro did produce fever when injected into normal animals, and had the characteristic delayed response of other immune fevers. There is more recent evidence for fever production mechanisms unrelated to the formation of antigen-antibody complexes in delayed hypersensitivity. In an effort to study fever in

"pure" delayed hypersensitivity, Atkins et al sensitized rabbits to various heterologous proteins and protein hapten conjugates and studied pyrogen production in vitro by various tissues in the presence of the conjugate and carrier. This was done at a time when negligible amounts of precipitating antibody were present. The results indicated that interaction of antigen with sensitized lymphocytes released a soluble substance, possibly a 'lymphokine', which stimulated pyrogen-producing cells to release endogenous pyrogen (57).

In Farr's original results with BSA injections in sensitized animals, febrile reactivity to injected antigen seemed to be correlated with the presence of detectable antibody, in that precipitating antibody was present in all animals responding with fever, and no animal without detectable antibody responded to injection with fever. However, the exact role of specific antibody was unclear since there were animals which had the antibody present in their serum but in whom no febrile response developed to the injected antigen (55).

Grey et al (67) provided the first evidence that fever due to injection of non-bacterial protein antigens was primarily a phenomenon of "immediate," or "humoral" hypersensitivity, by successfully inducing febrile reactivity in normal animals by passive transfer of specifically-immune serum to non-immune recipients. Five of seven recipients thus sensitized responded with fevers of 1°F or more when challenged with the BSA antigen. Passive transfer of non-

hyperimmune serum produced no such reactivity in the recipient, and there was no response in normal, non-immune animals given only BSA, thereby demonstrating the non-pyrogenicity of the BSA itself. However, spleen cell suspensions transferred to normal animals conferred no reactivity on the recipient when challenged. The results clearly implicated humoral, not cell-mediated, mechanisms.

The work of Mott and Wolff (56) continued to mount evidence for the significance of antigen-antibody interactions when they showed a rough correlation between the degree of temperature change and the antibody titer of specifically-sensitized animals challenged with an injection of antigen, in this case human serum albumin (HSA). They also noted that of the six rabbits which had been sensitized (according to the protocol used for all the animals), but in whom challenge with HSA elicited no febrile response, all six had undetectable antibody. Furthermore, febrile reactivity to injected antigen in normal animals passively sensitized with specifically immune serum was comparable in incidence to that seen in immune animals. However, of seven normal animals passively "sensitized" with the "immune" serum containing no demonstrable antibody, only one responded with fever to challenge.

Some clinical evidence to implicate the role of antigen-antibody interactions in the pathogenesis of fever was presented by Brittingham and Chaplin (68) who noted that febrile reactions to blood transfusions were associated with demonstrable antibodies against donor leukocytes in the

serum of those patients who had had both numerous (20 to 55) transfusions and repeated transfusion reactions. This group of patients responded with fever when injected with buffy-coat preparations of donor blood units, whereas the remaining red cell suspension of the donor blood resulted in no such response. This was compared to the responses of patients who had had fewer transfusions and no history of transfusion reaction. This group did not respond with fever to injection of either buffy coat or red cell suspension. Immune interactions were shown to be related to the fevers of certain clinical hemolytic disorders by Jandl and Tomlinson, who showed that hemolytic episodes due to immune hemolysis led to fever whereas hemolysis due to non-immune mechanisms had no such associated fever (69). Moreover, the degree of fever seemed to have no correlation to the degree of hemoglobinemia produced by different immune mechanisms, so fever correlated only with the presence of immune interactions, not with hemolysis *per se*.

The work of Root and Wolff (70) was the first to directly implicate antigen-antibody complexes as the specific pyrogenic mediator. They showed that antigen-antibody complexes prepared *in vitro* were regularly pyrogenic when injected into normal animals, a finding confirmed by Mickenberg *et al* (71).

The pattern of febrile response was used as an important differentiating characteristic in separating out the action of endogenous pyrogen from the less direct effects of the antigen or the antigen-antibody complexes. Unlike the

response to endogenous pyrogen, which has been shown to be a prompt, monophasic fever with a latent period of approximately 10 to 15 minutes (7), the fever curve after injection of antigen into sensitized animals was prolonged, with a longer latent period, a greater time interval to the peak of the response, and a greater total duration (72). These same response characteristics were observed by Mott and Wolff after injection by HSA (56), by Grey in passive transfer experiments, and by both Root and Wolff (70) and Mickenberg et al (71) in normal animals injected with antigen-antibody complexes.

The phenomenon of febrile tolerance proved to be a helpful tool to differentiate the pyrogenic effects of injected antigen from the effects of endogenous pyrogen to which tolerance has not been observed. Farr (72) noted originally that if sensitized rabbits which responded with fever to challenge with antigen were given repeated doses of the antigen, the febrile responses to the repeated injections became progressively smaller, with complete tolerance being achieved after 3 or 4 such injections. Several lines of evidence suggest that the phenomenon of tolerance to complexes differs from that of endotoxin tolerance, and may not be due simply to more rapid clearance from the circulation. Mott and Wolff (56) showed that the response to antigen in a sensitized animal was unaffected by a concomitant state of endotoxin tolerance in the animal, and that febrile tolerance to antigen could not be abolished with reticuloendothelial

blockade with Thorotrast. Furthermore, tolerance to antigen could not be passively transferred to sensitized animals, in clear distinction with endotoxin tolerance. Root and Wolff (70) also showed that the febrile response of normal animals to antigen-antibody complexes also was unaffected by a state of endotoxin tolerance. And Mickenberg et al (71) showed that tolerance to antigen-antibody complexes could persist when concomitant endotoxin tolerance in the same animal was reversed by reticuloendothelial system blockade with thorium dioxide. Thus, the pyrogenic effects of antigen in specifically-sensitized animals and of complexes in normal animals, can be readily differentiated from the direct effects of endogenous pyrogen and from the effects of contaminating endotoxin.

Root and Wolff, utilizing these phenomena, postulated a mechanism for the febrile reactivity to antigen injected into specifically sensitized animals. By challenging specifically sensitized rabbits with HSA and then collecting serum at 5 minutes after challenge and at 2 hours after challenge, they identified two pyrogenic substances in this serum by injecting these collections into normal, unchallenged animals. The late-appearing pyrogen, present in the serum of febrile donors at 2 hours post-challenge, had characteristics identical to that of endogenous pyrogen, and the serum from afebrile donors 2 hours post-challenge had no pyrogenic acitvity. However, the serum from donors (all of whom were afebrile) at 5 minutes post-challenge gave pyrogenic response

characteristics unlike that of endogenous pyrogen and more like that observed in sensitized animals challenged directly with antigen. They demonstrated that at any time in the first hour after injection of antigen into a sensitized animal, approximately 50 percent of the HSA was globulin bound, thereby suggesting that the HSA was present as circulating antigen-antibody complexes. It was the ability to produce febrile tolerance to injected antigen in a sensitized animal which enabled Root and Wolff to infer that their early appearing pyrogen was in fact antigen-antibody complex, and that the mechanism by which it produced fever was by release of endogenous pyrogen by the cells of the animal. By rendering complex tolerant- the rabbits which were given the early and late pooled serum collections, it was then observed that the late appearing pyrogen was able to mediate its effects in spite of complex tolerance. But the response of the early pyrogen in such complex tolerant animals was abolished. It was inferred that the early appearing pyrogen in the serum of sensitized animals challenged with antigen, which had a fever curve indistinguishable from that of antigen-antibody complexes, and which had no effect in animals previously rendered complex tolerant, was, in fact, antigen-antibody complex. And it was clear that the late appearing pyrogen, which had a fever response curve identical to that of endogenous pyrogen, and which was active despite complex tolerance, was indeed endogenous pyrogen. So it was postulated that the mechanism whereby protein antigen such as HSA

produced fever in actively or passively immune animals is by interaction with specific antibody to form antigen-antibody complexes which then interact with the cells of the host to stimulate the production and release of endogenous pyrogen. Grey et al (67) had postulated earlier that immune fever was probably mediated not directly by antigen or by complexes, but by a series of reactions in vivo since they noted the long lag phase of the immune fever curve and noted as well that the time required for antigen-antibody interactions could not account for that time delay.

In fever mediated by antigen-antibody complexes, it is now apparent that only certain classes of antibody are probably involved (56), that the structural characteristics of the complex play a role (73), and that interactions with other immune mechanisms, namely the complement system, may play a role (73).

There is evidence that precipitating antibodies mediate the pyrogenic response to antigen-antibody complexes. Farr in his original observations (55) noted that specific kinds of antibodies might be involved, although he (incorrectly) concluded that precipitating antibody was not the active factor in serum, because of the observation that some animals with demonstrable precipitating antibodies to BSA did not respond to the challenge of antigen. However, Root and Wolff (70) showed with complexes prepared in vitro that soluble complexes in antigen excess, prepared by solubilizing, with antigen, the washed precipitates formed at equivalence,

were as active in producing fever as were the soluble complexes prepared simply by adding antigen directly to the immune serum. Furthermore, the supernatants from the equivalence precipitation reactions were uniformly non-pyrogenic when injected. This evidence seems to clearly implicate the precipitating antibodies as the active participant in the febrile response to antigen-antibody complexes.

That specific immunoglobulin classes may be responsible for febrile reactivity can be inferred from the results of Jandl and Tomlinson (69) in their work on the fever of immune hemolytic episodes. It was observed that hemolysis due to "incomplete" anti-D antibodies produced significantly more fever than did hemolysis due to A-anti A or B-anti B interactions. The "incomplete" anti-D antibodies are from the IgG (Immunoglobulin G) class whereas anti A and anti B antibodies are IgM molecules (41). The significance of this class difference in relation to Jandl and Tomlinson's findings is significant in light of the evidence gathered by Mott and Wolff (56) that passive transfer of febrile reactivity to injected HSA was successful only with fractions of serum which were shown by immunoelectrophoresis to contain predominantly 7S globulin. That fraction which was rich in 19S macroglobulin produced no significant febrile reactivity in the passively 'sensitized' host. Thus, it seems that specific characteristics of different immunoglobulin classes may have importance in determining the role of any immunoglobulin in the pathogenesis of immune fever.

The macromolecular composition of immune complexes may play a significant role in producing fever. It is clear that in any given reaction mixture of antigen and antibody, whether in vitro (74) or in vivo (75), there is a heterologous mixture of different molecular species covering a broad range of molecular antibody to antigen ratios, including both free antibody and free antigen. And since these different molecular species seem to be handled by the host in different ways (75), and since they have different in vitro biological as well as physico-chemical characteristics (76), it is not surprising then that febrile reactivity to "antigen-antibody complexes" may represent the effects of one (or more) particular range or type of molecular species.

It was well documented by Arend and Mannik (76) that complexes of different macromolecular characteristics differ in their clearance from the circulation. They demonstrated as well that a given reaction mixture of antigen with antibody contains a broad range of macromolecular species, of varying antibody to antigen molecular ratios. By subjecting complexes of HSA-anti HSA prepared in 5 times antigen excess to a density gradient centrifugation they observed a variety of resultant peaks: a 6.6S peak representing unbound antibody and comprising approximately 20 percent of the total; and a broad spectrum of larger complexes from 14S to 22S which comprised approximately 46 percent of the total. Then by sequential timed sampling of the serum of a rabbit after injection of soluble complexes prepared

at five and at twenty times antigen excess, and ultracentrifugation of these samples, it was shown that there was a three phase exponential disappearance curve. There was a rapid initial exponential component represented by uptake of the large (greater than 11S) complexes. Other work showed that 99 percent of this uptake occurred in the liver (75). There was a second exponential phase representing rapid equilibration of the 11S complexes, followed by a third prolonged exponential phase corresponding to slow catabolism of the 11S and 6.6S components. Furthermore, the large (> 11S) complexes were most active in their in vitro complement fixing activity, since approximately ten times as many of the 11S complexes were required to fix an equivalent amount of complement. However, complement depletion of animals prior to injection of complexes had no effect on the rapid uptake of the large (11S) complexes. So it is clear that the in vitro activities of different species of complexes may not necessarily be of significance in determining their in vivo activities as it relates to clearance. And the authors concluded that the characteristics of the complexes which permit rapid clearance from the circulation parallel those which relate to complement fixation, but that the two characteristics are not necessarily functionally related in vivo.

Documentation that macromolecular composition of complexes may play a role in fever pathogenesis came when Root and Wolff (70) first produced fever in rabbits using HSA-

anti-HSA complexes prepared *in vitro*. They observed that complexes prepared at 10 times antigen excess (i.e. by adding to serum 10 times the amount of antigen required for equivalence) produced significant fevers consistently. Complexes prepared at 21 times antigen excess or at 3 times antibody excess produced lower and more variable responses. Mickenberg (73), however, showed that the structural characteristics of complexes may determine the degree of the fever response indirectly, through variable ability to fix complement. They showed that rabbits could be made febrile either by injection of HSA into an immunized animal or by injection of 10 times antigen excess complexes into a normal animal. However, treatment of these animals with cobra venom factor (resulting in complement depletion) prior to injection would abolish the responses of normal animals given the soluble complexes, whereas sensitized animals given antigen retained febrile reactivity. Since it was suspected that injection of antigen into a sensitized animal resulted in formation of large complexes like those formed at near equivalence or in antibody excess (75), complexes were prepared in 3 times antibody excess, and when injected into unsensitized animals, were pyrogenic even in cobra venom treated animals. Furthermore, animals could be made tolerant to 10 times antigen excess complexes by repeated injections of the complexes. (This treatment also produced partial complement depletion). However, such "complex tolerant" animals would still respond with fever when injected with the antibody excess complexes.

So it was apparent that a role for complement in the febrile response to soluble complexes prepared in 10 times antigen excess was possible. Also, complement depletion seemed to affect other host responses to injection of complexes: there was a decrease in the thrombocytopenia, a decrease in the duration of the leukopenia, and a marked reduction in the complement consumption observed as part of the response. Complement was further implicated by absence of an explanation of complex tolerance on the basis of more rapid clearance from the circulation or on the basis of a derangement of the effects of leukocyte endogenous pyrogen on the thermoregulatory center. That is, the only demonstrable change in complex tolerant animals was a (partial) depletion of complement. On the other hand, it was not clear that complement played any role in the response to the antibody excess complexes, since complement depletion by previous treatment with cobra venom factor did not alter the response, and since the response was not altered even in those animals which were tolerant to the soluble 20 times antigen excess complexes. This set of observations was especially interesting in light of the fact that the antibody excess complexes fixed complement in vitro much more rapidly than did the soluble antigen excess complexes and there was no significant difference in their in vivo complement consumption. However, the difference in the in vivo consumption may well be related to markedly more rapid clearance of the larger complexes, thereby de-

creasing the total exposure time to serum complement components.

So it seems clear that different *in vivo* and *in vitro* biological as well as physiochemical properties may play a complexly inter-related role in the mechanism or mechanisms of immune fever. And it seems obvious that some of these complexities might be sorted out by determining how the complexes interact with the cells of the host when some of these parameters such as duration of presence in the circulation, complement requirements, and macromolecular composition of the complexes could be controlled; that is, a study of the parameters governing the interaction of complexes with host cells *in vitro* would seem helpful. However, such studies have not been forthcoming and numerous attempts to stimulate cells *in vitro* with antigen-antibody complexes have been unsuccessful, using blood cells, Kupffer cells, hepatocytes, and mononuclear cells from spleen preparations (31). However, it seemed reasonable to attempt to stimulate monocytes in tissue culture, as the monocyte represents the probable cellular precursor of many tissue macrophages (34), and this preparation had been shown to be active with other stimuli such as heat-killed staphylococcus albus and endotoxin (Bodel, unpublished observations). Additionally, the preparation is relatively pure in that one can eliminate most of the polymorphonuclear leukocytes and most of the small lymphocytes (the chief contaminating cell type).

MATERIALS AND METHODS

Care of Materials and Animals

All glassware and instruments were rendered pyrogen free, after washing, by being heated in a dry oven at 170°C for at least 2 hours. Solutions and reagents were autoclaved at 120°C and 20 pounds pressure for at least 30 minutes.

New Zealand albino female rabbits, 6 kg in weight, were used as test animals. Temperatures were continuously recorded by a rectal thermistor probe connected to a continuous graph readout. The ambient temperature of the recording room was maintained at 68 to 72°F. Animals were restrained in stalls during temperature recording, and had been trained by at least two 3-hour periods in such stalls prior to assay injections.

Method of Injection and Recording of Temperatures

The rabbits' temperatures were allowed to equilibrate such that a steady baseline temperature recording of at least one hour's duration was obtained. Each animal was given an initial injection of normal saline prior to assay injections. All were given into a marginal ear vein. Since animals may be somewhat refractory to the effects of endogenous pyrogen during the hours following one profound febrile episode (77), injections into animals directly after high or prolonged fever was avoided. After a moderate febrile response, when the temperature had returned to its baseline value, at least one hour elapsed before the next

injection was given. Only those responses which were of rapid onset, monophasic type were considered as true endogenous pyrogen mediated responses (7). Maximum temperature change from baseline was used as the measure of febrile response. Occasionally, high fevers would be preceded by a slight, brief drop in temperature; in these instances, the maximum temperature rise was measured from the original baseline. In almost all experiments, pyrogenic testing of control and experimental samples was performed in the same group of rabbits, so that effects of variable responsiveness of individual rabbits on experimental results were minimized. Each rabbit received only 6 to 7 consecutive days of injections of human material, to prevent the complication of hypersensitivity fever (55).

Immunization of Rabbits and Collection of Hyperimmune Serum

Commercially prepared Bovine Gamma Globulin (BGG) (Armour Pharmaceutical Co., Chicago, IL) was dissolved in 0.1 Molar phosphate buffer, pH 7.0 to a concentration of 5 mg per ml. Eight ml of this solution was mixed with an equal volume of complete Freund's adjuvant, and 0.5 ml portions were injected into each footpad of 4 New Zealand albino rabbits. Booster injections of 1 mg of BGG were given intravenously four weeks later. Seven to ten days later, the animals were sedated with sodium barbital and bled by cardiac puncture. Some animals received another 1 mg of BGG intravenously 2 weeks after the first intravenous injection, and were bled subsequently. Samples of

all sera were cultured to confirm the absence of contamination, and were tested for specific antibody activity against BGG by Ouchterlony precipitation techniques. All sera were then pooled, divided into 5 ml aliquots, and frozen at -20°C. For the purposes of quantitative precipitation and formation of complexes for cell stimulation, 1:4 and 1:2 dilutions were made in 0.1 Molar phosphate buffer, pH 7.4

Iodination of BGG

A solution of BGG was labeled with a radioactive tracer for the purpose of determining the equivalence point of the pooled sera by quantitative precipitation technique. Conjugation with protein with I^{125} was accomplished according to the methods of Greenwood et al (78). To 10 microliters of a 5 mg/ml solution of BGG was added 3 mCi of I^{125} neutralized in 0.1 Normal HCl, and 20 microliters of Chloramine T (1.7 mg/ml in water) was added. This mixture was allowed to stand for 60 seconds and then the reaction was stopped by addition of 50 microliters of Sodium metabisulfite. This reaction mixture was then filtered through a Sephadex G-100 column equilibrate with 0.1 M phosphate buffer and successive 1 ml samples were collected. Aliquots of each tube were diluted and counted on a Nuclear Chicago scintillation counter. The first peak of radioactivity was saved, representing labeled protein rather than conjugated I^{125}.

Quantitative Precipitation of BGG with Rabbit Antiserum

In order to determine the zone of equivalence for the

pooled sera, a quantitative precipitation was carried out. An initial precipitation was first performed by adding 1.0 ml of 1:4 dilution of rabbit antiserum to varying amounts of a 1.0 mg/ml solution of BGG. These reaction mixtures were incubated at 37°C for approximately 10 minutes and then centrifuged at room temperature at 500 g for 20 minutes in an IEC Model PR-2 centrifuge. The supernates were decanted, and the precipitates washed with 0.1 Molar phosphate buffer, pH 7.4, followed by recentrifugation for a total of three such washings. Then the precipitates were solubilized in 0.5 ml of a 0.5 Normal solution of acetic acid, and made up to 20 ml by addition of water. Total protein concentration of these solutions were estimated by determination of O.D. at 280 millimicrons using a Coleman Model III spectrophotometer. These initial results indicated an approximate range of antigen concentration for the zone of equivalence to be around 0.6 mg BGG per 1 ml of 1:4 dilution of the rabbit antiserum.

A further precipitation test was then set up using BGG conjugated with I^{125} as a tracer. To variable amounts of a 1 mg/ml solution of BGG (.4 ml to .8 ml) was added 20 microliters of the solution of radioiodinated BGG. This mixture was well agitated on a Vortex mixer, and 1.0 ml of a 1:4 dilution of the pooled rabbit antiserum was added to each tube. These were quickly mixed on the Vortex mixer, incubated at 37°C for 30 minutes and then centrifuged at 500 g for 20 minutes. Supernatants were drawn off with a Pasteur

pipette and saved. Each pellet of precipitate was then gently resuspended in 5 ml of 0.1 M phosphate buffer, pH 7.4, and centrifugation repeated. This supernatant was drawn off with a Pasteur pipette and added to the original supernatant. This washing procedure was repeated twice more. Finally the washed pellet of precipitated complexes was solubilized in 0.5 ml of a 0.5 Normal acetic acid solution and diluted to a total of 20 ml with water; supernatants were also made up to a final volume of 20 ml.

A 1 ml aliquot of both supernatant and resolubilized precipitate was then counted for 1 minute in a Nuclear Chicago scintillation counter. The O.D. of the resolubilized precipitates was then determined in a Coleman III spectrophotometer at 280 millimicrons to determine the total protein content of the precipitate. The assumption was made that an O.D. reading of 1.5 units represented 1.0 mg/ml of protein. The amount of antibody precipitated in each tube was then calculated by subtracting the amount of antigen in the precipitate, determined by the radioactivity of the solubilized precipitate, from the total protein content of the precipitate as determined by the O.D. reading. Assumptions were made that the small amounts of radioiodinated BGG were negligible in relation to the amount of unlabeled antigen, and that labeled and unlabeled BGG were well mixed and behaved identically in the process of combining with the antibody. The equivalence point was chosen as that dose of antigen which precipitated the maximum amount of antibody.

Preparation of Complexes

Soluble immune complexes in 10 times antigen excess were prepared *in vitro* by adding 10 times the amount of antigen required for equivalence to a given amount of a 1:2 dilution of antiserum in a small test tube. The mixture was immediately agitated on a Vortex mixer and then incubated for approximately 1/2 hour at 37°C. The mixtures were then centrifuged at room temperature, at 1000 g for 20 minutes. The supernatants were then decanted and added directly to tissue culture flasks containing the prepared monocytes. Complexes in 10 times antibody excess were similarly prepared by adding 10 times the amount of antibody required for equivalence to a given amount of antigen. Similarly prepared complexes were formed at 50 times antigen excess. The dose of complexes given to cells was varied by changing the concentration of BGG and the dilution of rabbit antiserum used in forming the precipitates. This method of varying the dose of complexes was chosen in order to maintain a constant volume of stimulus added to the tissue culture flasks, thereby eliminating the possible effects of differential dilution of the culture medium by different stimuli. In cases where flasks were to receive no stimulus or a colume of stimulus material less than 1.0 ml in volume, the additional volume was added in the form of 0.1 Molar phosphate buffer, pH 7.4.

Precipitated complexes at equivalence and at 3 times antibody excess were prepared by adding the appropriate

amounts of antigen and antibody, incubating and centrifuging the mixture as above, and then decanting the supernate. The pellet was then gently resuspended in phosphate buffer (.1 Molar at pH 7.4) and recentrifuged at 1000 g for 10 minutes. This washing was repeated twice, the final supernatant decanted, and the pellet was resuspended in an amount of phosphate buffer equal to the total initial reacting volume, with agitation on a Vortex mixer. Samples of this suspension of antigen-antibody complex particles were added directly to the tissue culture flasks of the monocytes.

Isolation of Peripheral Monocytes

Human peripheral blood monocytes and lymphocytes were isolated according to the method of Boyum (30). All procedures were performed with sterile, pyrogen free glassware and solutions, and utilizing sterile technique throughout.

Twenty ml of 50% Hypaque (sodium diatrizoate), 70.6 ml of a 9% aqueous solution of Ficoll (a large polysaccharide polymer, molecular weight approximately 400,000, from Pharmacia Fine Chemicals, Upsala, Sweden), and 9.4 ml of water were combined and 8 ml aliquots of this Hypaque-Ficoll solution were placed in 40 ml conical glass centrifuge tubes.

Peripheral venous blood from healthy human volunteers was drawn directly from venipuncture into a large heparinized syringe (10 units/ml heparin). An additional 30 ml of unheparinized blood was drawn fro serum. Approximately 120 ml

of blood was mixed in a large flask with approximately 260 ml of saline and stirred slowly with a magnetic mixer while 32 ml aliquots were gently layered over each 8 ml aliquot of the Hypaque-Ficoll solution. These tubes were then centrifuged at room temperature, at 400 g, for 40 minutes. After centrifugation red cells and PMNs were found at the bottom of the tubes. Approximately 1 to 2 cm above the upper border of this button was a white ring containing monocytes and lymphocytes. Above this ring was approximately 30 ml of clear solution. This top layer of solution was removed by suctioning gently through a Pasteur pipette; the layer of monocytes and lymphocytes was then carefully removed with a 5 ml pipette with a pipette bulb attached. It proved helpful to scrape the sides of the glass with the tip of the pipette to remove attached cells. The rings from two such centrifuge tubes (approximately 10-12 cc) were combined in a new 40 cc conical centrifuge and diluted with 30 cc of a solution of Krebs-Ringer Phosphate buffer (KRP) containing 10 units/ml of heparin. After thorough mixing with a 10 ml pipette the tubes were centrifuged at room temperature, at 150 g, for 15 minutes, the wash supernatant discarded by suctioning, and the cells gently resuspended in 0.5 to 1 ml of tissue culture medium (Minimal Eagles Medium with 50 units per ml of penicillin and streptomycin, and 200 millimolar L-glutamine). All the cells were collected to make a pool or approximately 8 ml of cells in tissue culture medium. A white blood cell

count was performed on a Coulter cell counter Model Z_f (Coulter Electronics, Hialeah, FL). A small drop of the cell suspension was placed on a coverslip with a drop of albumin and smears were made for differential cell count. A total of 120 ml of peripheral blood usually yielded 15 to 20 X 10^7 cells of which 20 to 35% were monocytes, the remainder being lymphocytes. Granulocytes accounted for 0 to 1% of the cells thus isolated. In some experiments, numbers of monocytes were confirmed by incubation of 0.2 ml of the cell suspension at 27°C for two hours with a drop of a suspension of heat killed staphylococci (3 X 10^9 particles per ml). These cells were then suspended in 30 ml of normal saline, centrifuged at room temperature, at 400 g, for 15 minutes. The supernate was decanted and the cell pellet suspended in a drop of albumin; coverslip smears made prepared, stained with Wright's stain and examined under oil. Cells containing staphylococci were counted. Numbers of phagocytic cells compared well with estimates of monocyte count made by morphology.

Incubation of Cells

Tissue culture flasks were prepared containing approximately 2 X 10^7 cells (approximately 4 to 6 X 10^6 monocytes) in a total volume of 6 ml MEM with 15% homologous serum. Flasks were then gassed with 5% CO_2 in air, blown through a sterile, cotton plugged Pasteur pipette, and incubated at 37°C for 3 hours. At this time, when the monocytes were observed to have attached to the bottom of the flask, the

medium and floating cells (i.e. lymphocytes) were removed. Four ml of fresh medium, with 20% serum, was then gently added to the flasks, followed by 1 ml of experimental or control solutions. The final concentration of serum in the flask remained 15%. The flasks were then again gassed with 5% CO_2 in air and incubated overnight (16 to 18 hours) in a 5% CO_2 incubator. After examination of the cells under a 20X tissue culture microscope, the supernatants from the flasks were then transferred to centrifuge tubes, centrifuged at 1000 or 2000 g. The higher speed was used for supernatants containing staphylococcal particles. The supernatants were then drawn up into syringes; two to three drops of each supernatant was added to tubes of thioglycolate broth and cultures incubated for at least 3 days to assure sterility. Data from injection of any material which proved to be contaminated was excluded from the final results.

In each experiment in which cells were obtained from a single donor, supernatants from all experimental flasks, usually six to nine, were tested for pyrogen in the same rabbits. Usually supernatant from a single flask provided two test injections which were given to two different animals.

Analysis of Data

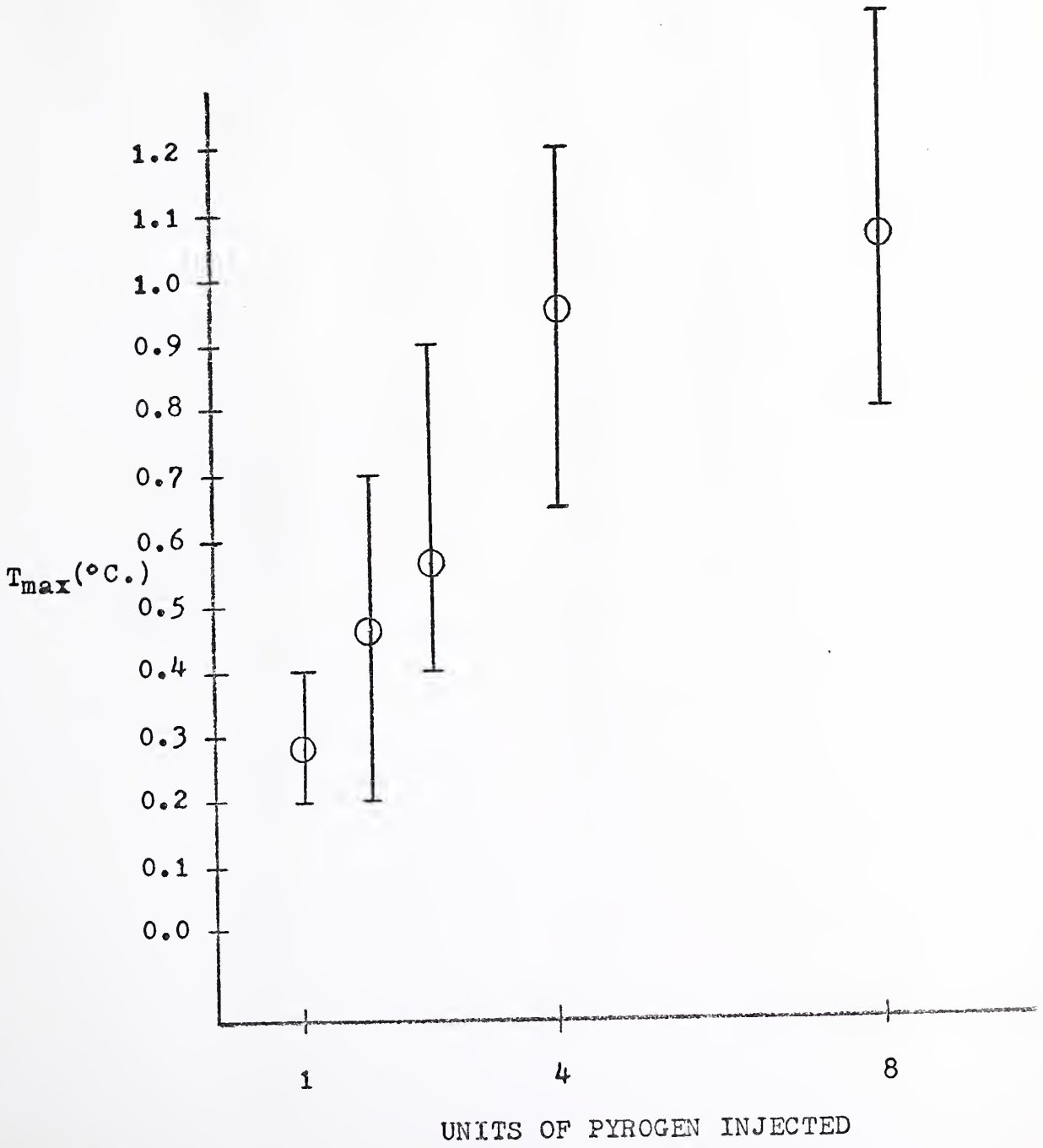
In order to convert results of pyrogen assays to numbers with linear scale parameters, maximum temperature changes (Δ T max) were converted to units of pyrogen dose. The

rationale and techniques of this procedure is as follows. When increasing doses of a preparation of human monocyte pyrogen were injected into a group of rabbits, a lagarithmic function was observed. (Fig. 1) In the range of 0.3°C to 0.9°C, Δ T max increases almost proportionally with dose, but a plateau is reached after which only small increases in Δ T max can be observed in response to relatively large increases in the amount of pyrogen injected. Similar response characteristics have been observed previously for human and rabbit granulocyte pyrogen (77, Bodel, unpublished observations). When the monocyte pyrogen response curve is plotted as a function of the logarithm of the dose of pyrogen administered, and the results analyzed by statistical tests for trend (79), a linear function best describes the curve. The best line describing the curve was determined by the method of least squares. When experimental data was analyzed, individual Δ T max values were converted to pyrogen dose units by use of this curve. A single pyrogen dose unit was defined as the amount of pyrogen required to effect a Δ T max of 0.3°C, a minimal positive febrile response. The range of pyrogen units extended from 1.0 to approximately 8.0 units. The scale of pyrogen doses was set to comply with this definition of a minimal febrile response, and all Δ T max values were then converted to some constant multiple of the minimal pyrogenic dose unit. Therefore any response of Δ T max equal to 0.3°C or greater corresponds to a pyrogen dose of 1.0 or greater, and is interpreted to

FIGURE 1.

DOSE RESPONSE CURVE FOR
HUMAN MONOCYTE PYROGEN

AVERAGE VALUES WITH
RANGES OF RESPONSES



be a significant amount of pyrogen. Any Δ T max of less than 0.3°C corresponds to a pyrogen dose of less than 1.0 and probably represents an insignificant amount of pyrogen. Any response which was not included within the range of the dose response curve was excluded as being uninterpretable. In the figures below, the data is presented as the statistical mean of at least 14 individual values, reported with confidence limits determined by the two-tailed t test (80). The number of actual observations made is also included.

RESULTS

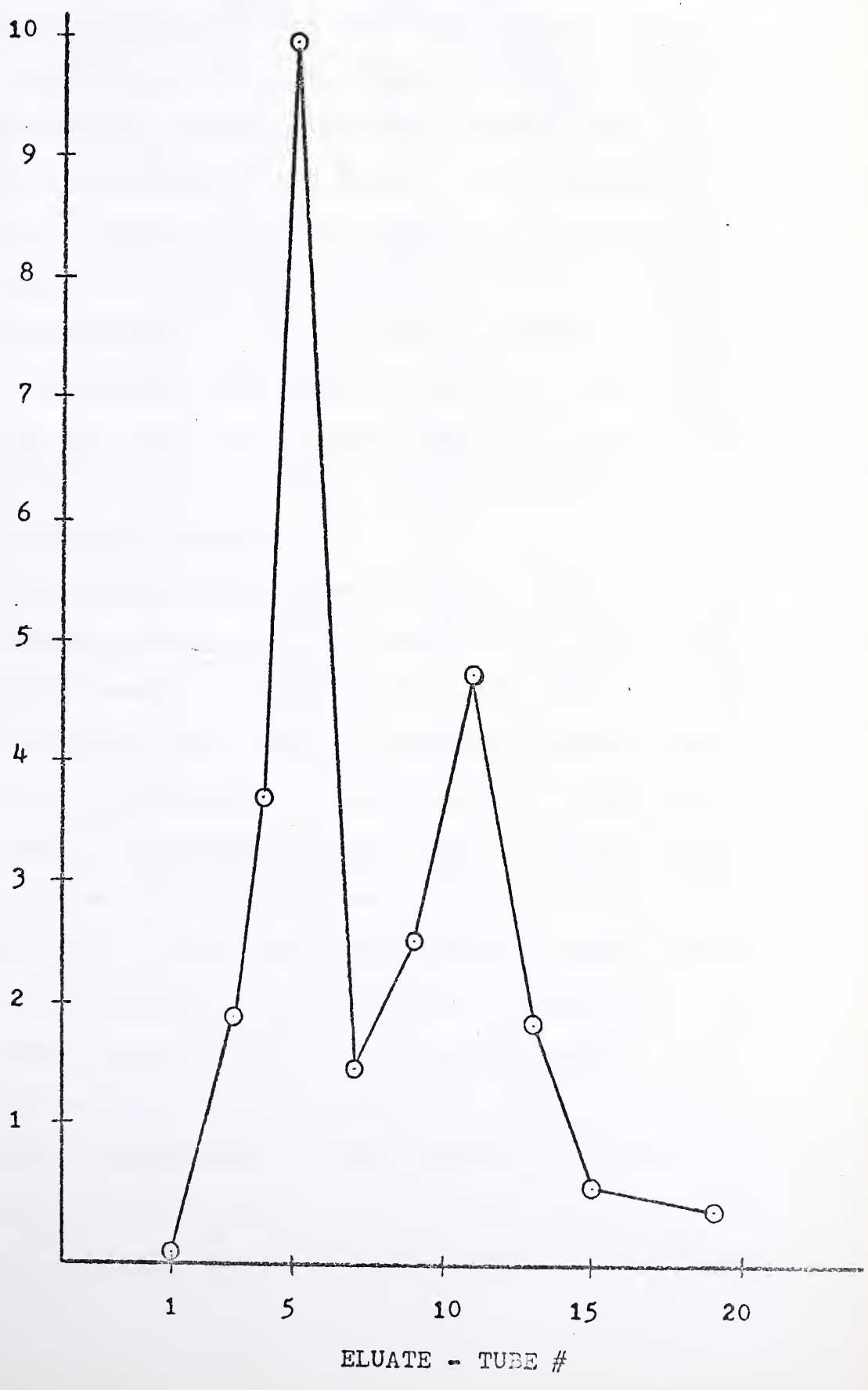
Preparation of I^{125} Iodinated BGG

In preparation for a quantitative precipitation test iodination of BGG with I^{125} was performed. After column chromatography of the reaction mixture in a Sephadex G-100 column, two peaks of radioactivity were obtained, as shown in Fig. 2. As shown by others, the first peak includes the iodinated protein, and the broader second peak represents unconjugated radioactive iodine (78).

Determination of the Equivalence Point

A series of quantitative precipitations were performed in order to determine the relative amounts of pooled antiserum and BGG required for equivalence. A preliminary precipitation using only non-iodinated BGG showed that maximum precipitation occurred when 1 ml of a 1:4 dilution of the pooled antiserum was reacted with approximately .6 mg of BGG (0.6 ml of a 1 mg/ml solution). In order to further define the range of equivalence, precipitations were done using BGG to which 20

FIGURE 2.
I^{125} IODINATION OF BOVINE GAMMA GLOBULIN.
ACTIVITY OF THE CHROMATOGRAPHED ANTIGEN.

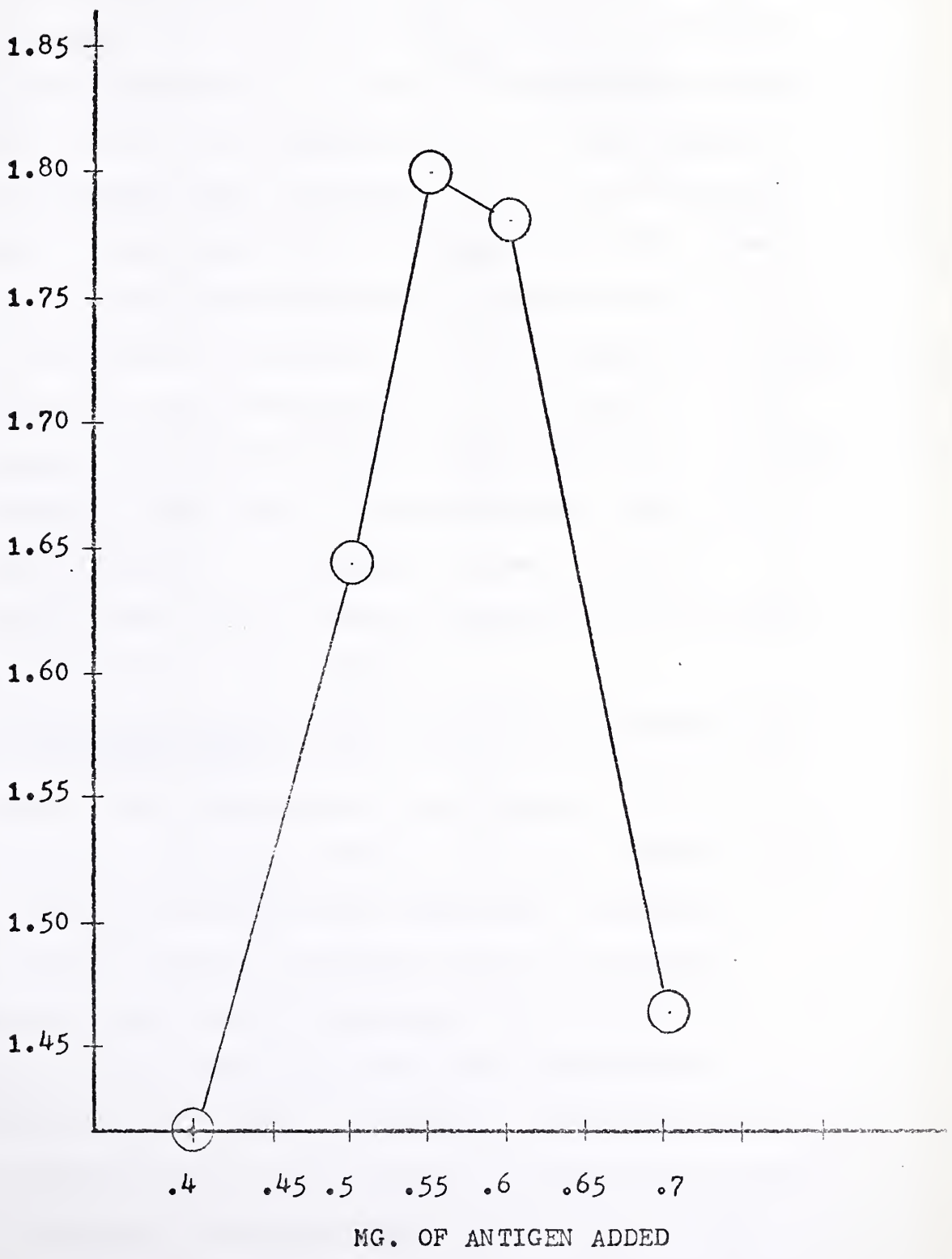


of I^{125} iodinated BGG had been added. The relative amounts of antigen and antibody in the supernatant and in the precipitate were determined as described in Methods. Equivalence was defined as the point at which the maximum amount of antibody was precipitated. In Fig. 3, the milligrams of antibody precipitated is plotted against the amounts of antigen added to each reaction mixture. Maximum antibody was precipitated when 0.55 mg of BGG was reacted with 1.0 ml of a 1:4 dilution of the rabbit antiserum. This determination was then used to calculate amounts of antigen and antibody needed to form complexes of varying ratios in antigen or antibody excess.

Additional quantitative precipitations were done with reaction mixtures containing 10 times antigen excess and 10 times antibody excess to examine the total amount and composition of protein in the soluble complexes present in the supernates of such mixtures. This was done to determine whether marked differences in total dose of soluble complexes would result from the two reaction mixtures. It was determinted that, in a 10 times antibody excess mixture, approximately 64% of the antigen remained in the supernatant: and in a 10 times antigen excess mixture, approximately 71% of the antibody remained in the supernatant. These findings indicated that supernatants of these reaction mixtures contained roughly similar amounts of protein, although it is likely that the exact molecular species present would differ markedly (74).

FIGURE 3.

QUANTITATIVE PRECIPITATION.
EQUIVALENCE RANGE OF RABBIT
ANTISERUM & I^{125} LABELED BGG



Temperature Responses of Rabbits to Human Monocyte Pyrogen

Monocytes were incubated in tissue culture flasks overnight with heat killed staphylococci, and the supernatant was injected into rabbits. A typical temperature recording is shown in Fig. 4. The characteristics of this febrile response resembles those previously reported for leukocyte endogenous pyrogen, both human and rabbit (1, 25). There was a latent period after injection of approximately 15 minutes, followed by a monophasic rise in temperature peaking within or at one hour and returning to the baseline within 1 to 2 hours. Rabbits with higher peak temperature responses usually required more time to return to their normal baseline. No differences in the shape of the curve were apparent when other kinds of stimuli were used to initiate pyrogen production by these cells.

Pyrogen Production by Monocytes Incubated with Soluble Complexes in Antigen Excess

Monocytes were incubated in the presence of soluble antigen-antibody complexes formed in 10 times antigen excess. Control flasks included one with monocytes incubated in medium without antigen or antibody, one with monocytes and antiserum, and one with monocytes and antigen. In addition, a flask containing heat killed staphylococci was included to provide a positive control. The results of one such experiment are shown in Fig. 5. Each curve represents the average temperature response to 2 or more test animals. Whereas control injections produced little or no fever, injection of supernatants from cells plus soluble

FIGURE 4

RABBIT TEMPERATURE RESPONSE TO
MONOCYTE ENDOGENOUS PYROGEN

TEMPERATURES REPRESENT THE AVERAGE
VALUES OF TWO RABBIT RESPONSES TO INJECTION
OF THE PRODUCT OF APPPOXIMATELY
2×10^6 MONOCYTES

FIGURE 4.

RABBIT TEMPERATURE RESPONSE TO MONOCYTE ENDOGENOUS PYROGEN.

○ - ○ UNSTIMULATED MONOCYTES

□ - □ MONO'S & STAPHYLOCOCCI

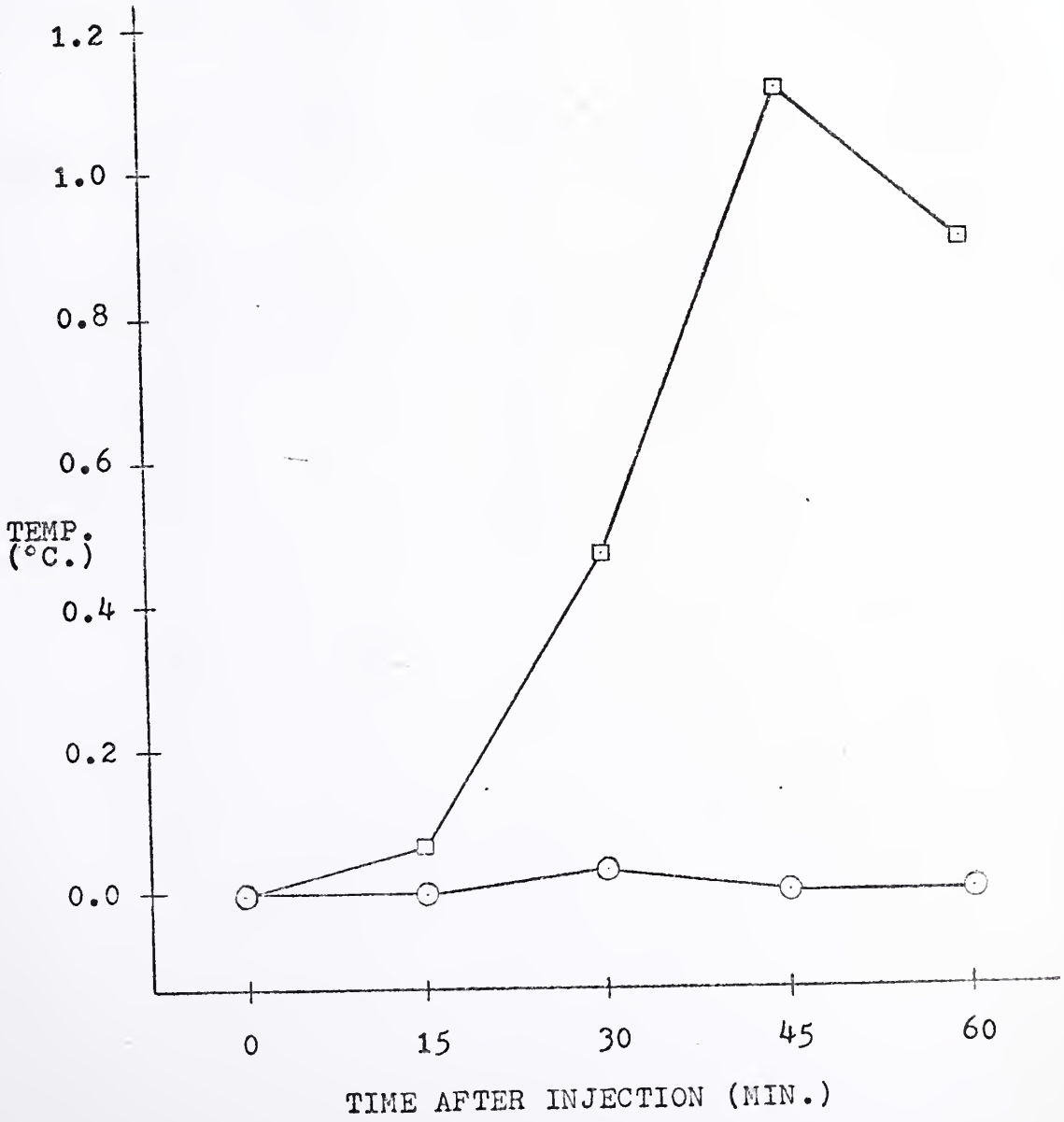


FIGURE 5

RABBIT FEVER RESPONSE TO MONOCYTE
PYROGEN PRODUCED IN RESPONSE TO
ANTIGEN-ANTIBODY COMPLEXES

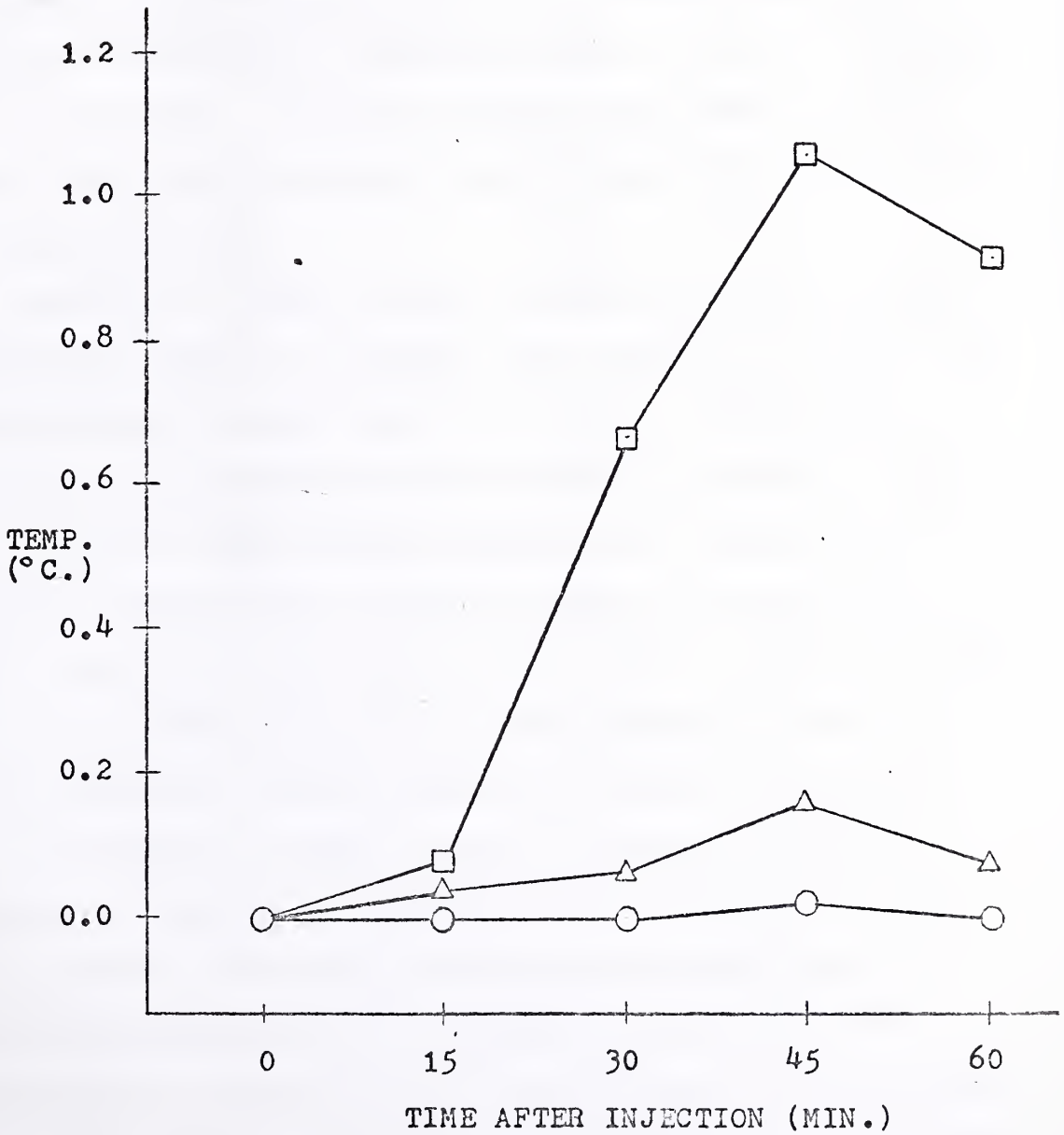
TEMPERATURES REPRESENT THE AVERAGE
VALUES OF TWO RABBIT RESPONSES TO INJECTION
OF THE PRODUCT OF APPROXIMATELY
2×10^6 MONOCYTES

FIGURE 5.

RABBIT FEVER RESPONSE
TO MONOCYTE PYROGEN.

STIMULATION OF MONO'S
BY SOLUBLE COMPLEXES IN
10 TIMES ANTIGEN EXCESS.

○—○ MONOCYTES ALONE
△—△ MONOCYTES WITH BGG.
OR WITH ANTISERUM.
□—□ MONOCYTES WITH IMMUNE
COMPLEXES.



complexes produced clear, reliable febrile responses.

A summary of the data from 14 experiments of this kind is presented in Fig. 6. As shown in bar A, monocytes incubated without any added material produced insignificant amounts of pyrogen. Since a minimal positive pyrogenic response (ΔT max of 0.3°C) corresponds to a pyrogen dose unit of 1.0, the mean value for monocytes alone, is 0.6 $\pm$ .12 units ($p < 0.001$, n=24). That is, there is less than a 0.1% chance that monocytes alone produce significant amounts of endogenous pyrogen.

Similarly as shown in bar B, monocytes do not produce significant amounts of pyrogen after addition of either BGG or antiserum, in doses equal to or greater than those present in the preparations with soluble complexes. The mean value for these pyrogen doses (taken together to provide a statistically adequate number of trials), is 0.72 $\pm$ .06 units ($p < 0.001$, n=14). That is, there is less than a 0.1% chance that cells in the presence of BGG or rabbit antiserum produce significant amounts of pyrogen.

By contrast as shown in bar D, addition of soluble complexes to monocytes stimulates the production of endogenous pyrogen. The value of the mean pyrogenic dose for these supernatants is 2.3 $\pm$ .88 units ($p < .001$, n=36), significantly greater than 1.0. Heat killed staphylococci were also potent stimuli of pyrogen production by monocytes (see bar E). The mean pyrogenic dose was 3.3 $\pm$ .79 units ($p < 0.05$, n=17). This corresponds to a mean temperature

FIGURE 6

MONOCYTE PYROGEN PRODUCTION BY DIFFERENT CELL STIMULI (IN PYROGEN DOSE UNITS)
1 UNIT PYROGEN $\triangleq$ 0.3°C Δ T MAX

A. MONOCYTES ALONE - CONTROL

B. MONOCYTES WITH BGG ALONE OR WITH ANTISERUM ALONE - CONTROL

C. ANTIGEN-ANTIBODY COMPLEXES ALONE, WITHOUT CELLS - CONTROL

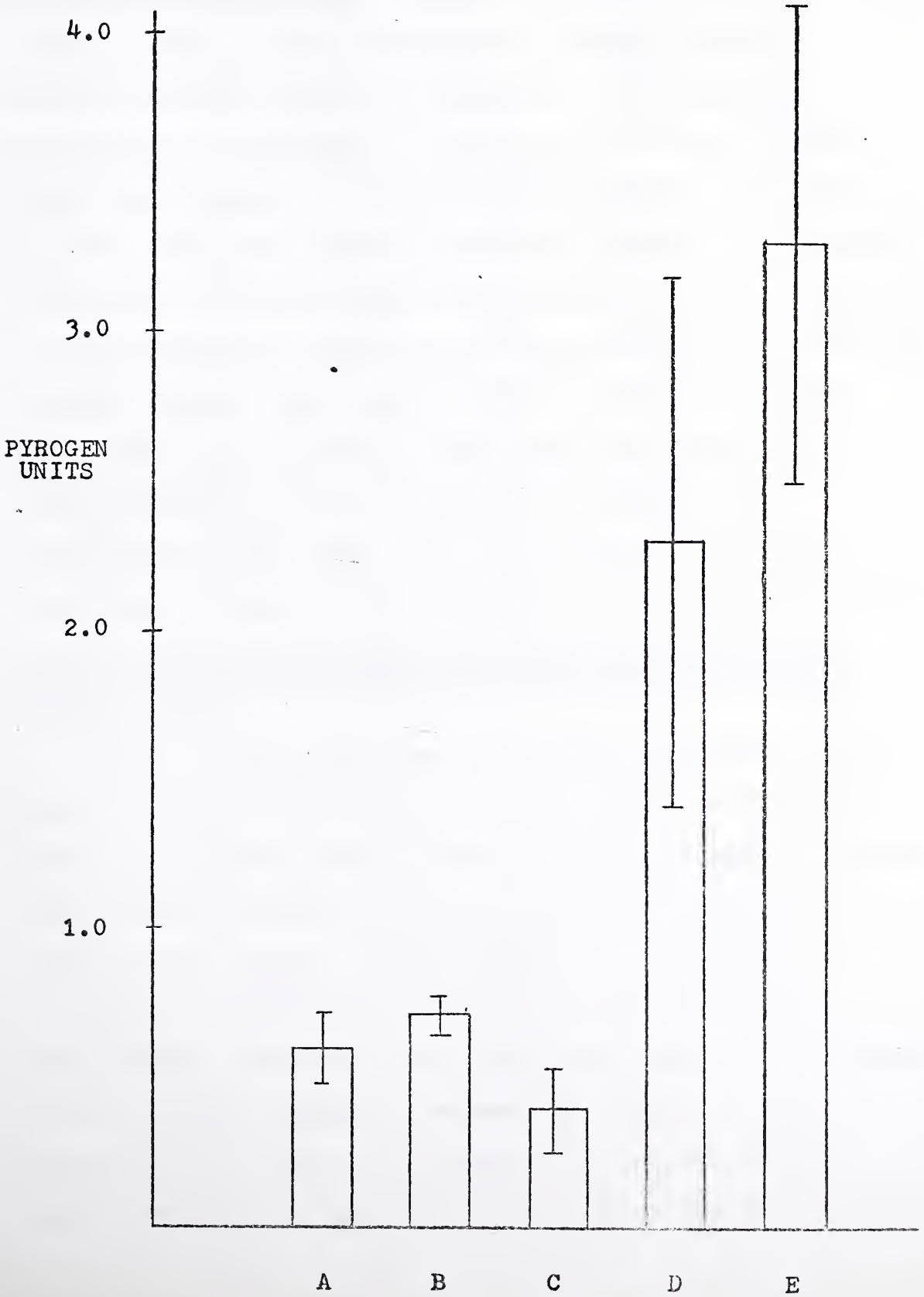
D. ANTIGEN-ANTIBODY COMPLEXES IN 10 TIMES ANTIGEN EXCESS (HIGH DOSE) WITH MONOCYTES

E. MONOCYTES WITH HEAT-KILLED STAPHYLOCOCCI

BARS REPRESENT STATISTICAL MEANS WITH 99% CONFIDENCE LIMITS, AND REPRESENT THE PRODUCT OF APPROXIMATELY 2 X 10^6 MONOCYTES

FIGURE 6.

MONOCYTE PYROGEN PRODUCTION
BY DIFFERENT CELL STIMULI.



response somewhere in the range of 0.67 to 0.84°C.

In order to demonstrate that the pyrogen present in supernatants from tissue culture flasks containing cells and complexes was, in fact, endogenous pyrogen, and not antigen-antibody complexes themselves, the following experiment was performed. Tissue culture flasks without cells were prepared containing tissue culture medium and soluble complexes. These flasks were incubated and treated identically to other flasks which contained cells. Injections of the centrifuged supernatants were uniformly non-pyrogenic, the mean pyrogen dose being 0.4 ± .14 units ($p < 0.001$, n=15) (see Fig. 6, bar C). Thus the pyrogen present in flask supernates after incubation of complexes with monocytes was most likely due to a substance produced by the cells in tissue culture rather than the complexes alone.

Pyrogen Production by Monocytes Incubated with Varying Doses of Soluble Antigen-Antibody Complexes in 10 Times Antigen Excess

To test whether the amount of pyrogen produced by the monocytes was directly related to the number of complexes added, equivalent numbers of cells in culture were stimulated with different amounts of soluble complexes prepared in 10 times antigen excess. Equal volumes were maintained by diluting the solutions of antigen and antibody before preparing the complexes. The lower dose was given by adding 1 ml of soluble complexes prepared by mixing a 1 mg/ml solution of BGG with a 1:4 dilution, in buffer, of the rabbit antiserum, or by adding 0.5 ml of buffer plus 0.5 ml

of the high dose solution. The high dose solution was given by adding 1 ml of soluble complexes prepared by mixing a 2 mg/ml solution of BGG with a 1:2 dilution of the rabbit serum. As seen in Fig. 7, bars B & C, there is a positive correlation between the concentration of complexes added to the monocytes and the amount of pyrogen produced. The low dose of complexes stimulated production of 1.5 $\pm$ .18 units of pyrogen ($p < 0.01$, n=23), while the higher dose of complexes stimulated production of 2.3 $\pm$ .67 units ($p < 0.01$, n=36). There is less than a 1% chance that the two doses stimulate production of equivalent amounts of pyrogen.

Comparison of Production of Monocyte Pyrogen in Response to Qualitatively Different Antigen-Antibody Complexes

Since complexes of varying size and differing antibody to antigen ratio are known to have different *in vivo* and *in vitro* biological activities, the effects of soluble complexes prepared in 10 times antibody excess and in 50 times antigen excess were compared to those of the 10 times antigen excess complexes. The results are presented in Fig. 8. Soluble complexes in 10 times antibody excess stimulated production of 1.9 $\pm$ .90 pyrogen units ($p < 0.01$, n=18). This finding indicates that soluble complexes in antibody excess are also capable of stimulating cells. Although this figure is lower than the one for 10 times antigen excess, direct comparison of the 10 times antibody excess complexes with the 10 times antigen excess complexes seems not justified, since differences in pyrogen production follow alteration in dose of complexes (see above), and it

FIGURE 7

DOSE RELATIONSHIP OF PYROGEN PRODUCTION TO CONCENTRATION OF ANTIGEN EXCESS COMPLEXES
(IN PYROGEN DOSE UNITS)
1 UNIT PYROGEN = 0.3°C ΔT MAX

A. MONOCYTES ALONE - CONTROL

B. MONOCYTES WITH HIGH DOSE COMPLEXES

C. MONOCYTES WITH LOW DOSE COMPLEXES
(1/2 OF THE HIGH DOSE CONCENTRATION)

BARS REPRESENT STATISTICAL MEANS WITH 99% CONFIDENCE LIMITS AND REPRESENT THE PRODUCT OF APPROXIMATELY 2 X 10^6 MONOCYTES

FIGURE 7.

DOSE RELATIONSHIP OF PYROGEN PRODUCTION TO CONCENTRATION OF ANTIGEN EXCESS COMPLEXES.

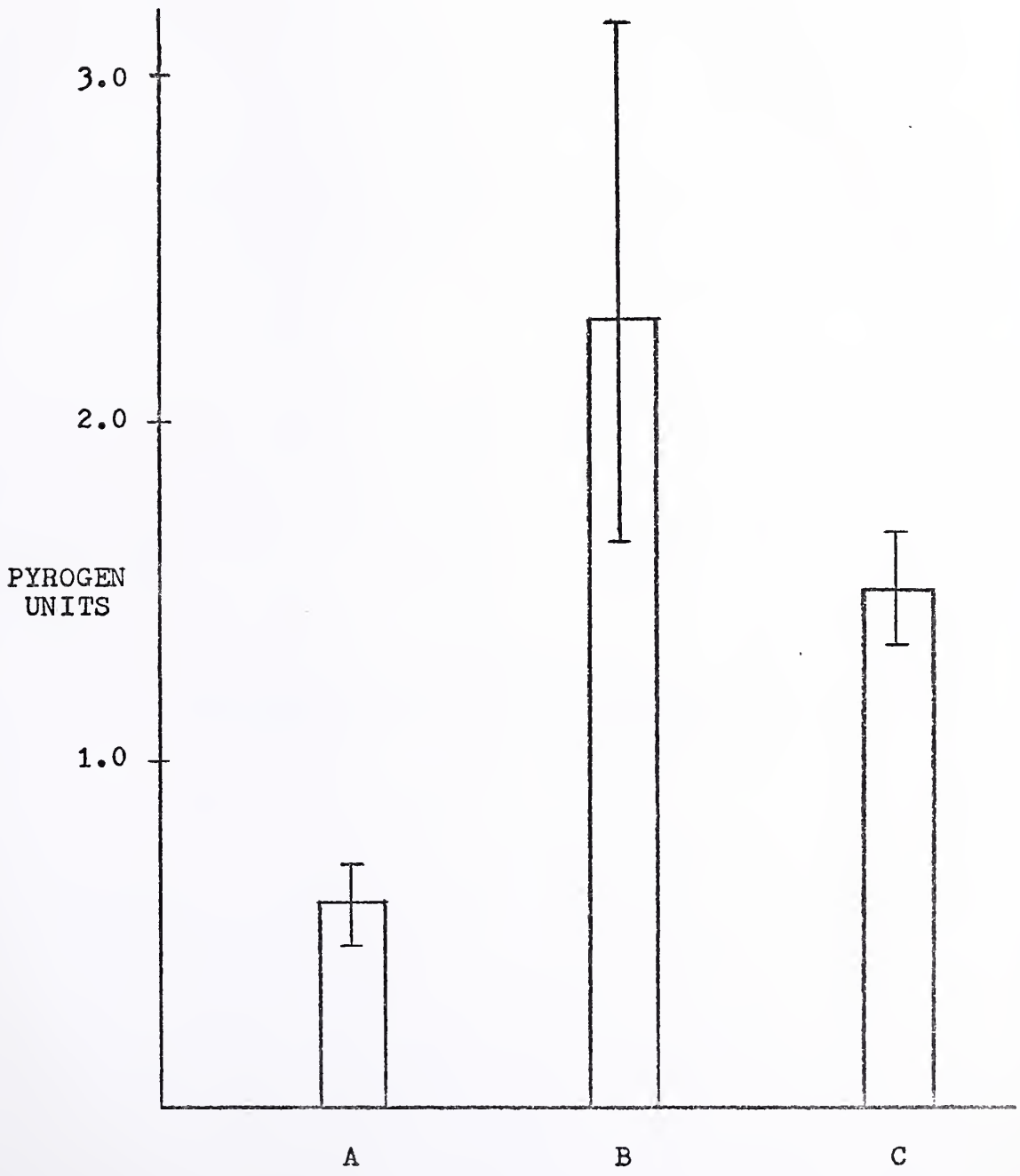


FIGURE 8

MONOCYTE PYROGEN PRODUCTION IN RESPONSE TO IMMUNE COMPLEXES OF DIFFERENT ANTIBODY: ANTIGEN RATIOS. SOLUBLE AND PRECIPITATED COMPLEXES

A. 10 TIMES ANTIGEN EXCESS

B. 10 TIMES ANTIBODY EXCESS

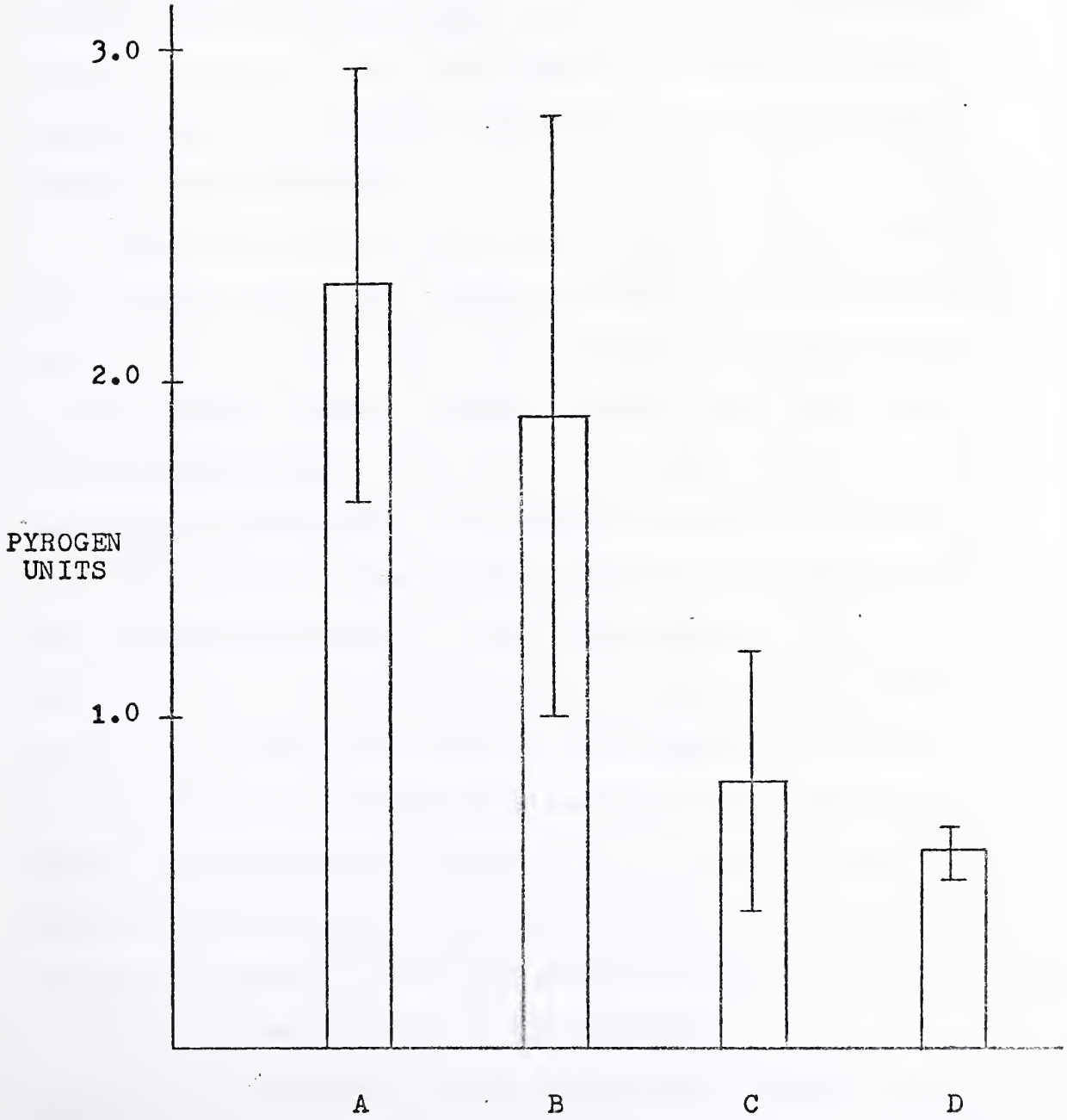
C. 50 TIMES ANTIGEN EXCESS

D. COMPLEX PRECIPITATES

BARS REPRESENT STATISTICAL MEANS WITH 95% CONFIDENCE LIMITS, AND REPRESENT THE PRODUCT OF APPROXIMATELY 2 X 10^6 MONOCYTES

FIGURE 8.

MONOCYTE PYROGEN PRODUCTION IN RESPONSE TO IMMUNE COMPLEXES OF DIFFERENT ANTIBODY:ANTIGEN RATIOS. SOLUBLE AND PRECIPITATED COMPLEXES.



was not possible to accurately quantitate numbers of complexes in each system. However, it was noted that a mixture of 10 times antibody excess complexes produced a significantly larger pellet of precipitate than did the 10 times antigen excess complexes. Thus, the amount of complexes present in soluble form was probably less than that in the 10 times antigen excess mixture.

Complexes prepared in 50 times antigen excess were also added to the monocytes, and pyrogenicity of the supernatants determined. In this case, the amount of complex present in the soluble form was clearly greater than that found in the 10 times antigen excess mixture. However, stimulation of pyrogen production by the soluble complexes prepared at 50 times antigen excess was not clearly demonstrated; the mean pyrogen dose was 0.8 ± .17 units ($p < 0.4$, $n=14$), Fig. 8, bar C. This indicates that there is an approximately 80% probability that the mean amount of pyrogen produced was not a significant amount. The response was clearly lower than the response to 10 times antigen excess complexes, even though there were at least as many soluble complexes present at 50 times antigen excess, since there was virtually no precipitate in this mixture.

Response of Monocytes to Antigen-Antibody Complex Precipitates

Since monocytes produce pyrogen after simple phagocytosis of particles such as heat killed staphylococci, it was necessary to be sure that the responses observed with soluble complexes were not due to phagocytosis of particles

of precipitated complexes which might remain after centrifugation or which might form inadvertently in the flasks. In addition, the effect of precipitates of antigen-antibody complexes on monocyte pyrogen production was of interest. Therefore, precipitates formed at equivalence, at 3 times antibody excess, and at 10 times antibody excess were prepared, washed 3 times to remove any remaining soluble complexes, resuspended in phosphate buffer, and added to the standard cultures of monocytes. The results of these supernatant injections are shown in Fig. 8, bar D. Immune precipitates uniformly failed to induce pyrogen production. The mean pyrogen dose was 0.6 $\pm$ .08 units ($p < 0.001$, n=19). In order to ensure that the precipitates had not injured the cells, preventing them from producing pyrogen, an experiment was included in which heat killed staphylococci with and without precipitates were added to some cells. In this case, a large amount of pyrogen was produced, both in the presence and absence of precipitates, in response to the staphylococci. Thus there was no evidence that the precipitates inhibited pyrogen production.

Microscopic Observations of Monocytes in Tissue Culture

Tissue culture flasks were usually examined, after incubation for approximately 18 hours, under a tissue culture microscope at 20X magnification. The observations may be summarized as follows. Monocytes incubated in medium alone appeared as cells which were fixed to the flask surface, with minimal numbers of cells floating free in the

medium; cells were separate and unclumped, round or oval, and displayed a minimal granular appearance to the cytoplasm with occasional ameboid shapes with cytoplasmic extensions. Cells that had been stimulated by either staphylococci or antigen-antibody complexes in 10 times antigen or antibody excess appeared somewhat larger, most still fixed to the surface, but often with significant numbers of floating, single cells or cell clumps. Cells fixed were often clumped together and were usually round. A more heavily granular appearing cytoplasm was evident in most cells, fixed and floating. In general, these changes of clumping and granularity were produced to a greater degree by stimuli in the following order: staphylococci > high dose soluble, 10 times antigen excess complexes > soluble 10 times antibody excess complexes > low dose soluble 10 times antigen excess complexes. In general, soluble, 50 times antigen excess complexes produced only minimal changes. Negligible changes were observed with BGG alone or with antiserum alone. However, resuspended precipitates were found to produce marked changes as described above, with large masses of floating cell clumps as well as some fixed cell clumps. Because more accurate microscopy was not performed the exact relationship of these changes to specific alterations in cell morphology is not known.

CONCLUSIONS AND DISCUSSION

The results of these experiments indicate that immune complexes can interact with pyrogen producing cells to

stimulate the production of endogenous pyrogen. While Root and Wolff (70) have shown previously that injection of antigen into a sensitized animal was associated with the appearance of endogenous pyrogen in the circulation, the *in vivo* pathways, presumably involving interactions of complexes with some cells, were unknown. Atkins *et al* attempted to stimulate blood cells, predominantly polymorphonuclear leukocytes, with complexes of rabbit antiserum and BGG-DNP, and failed to generate any detectable pyrogen (57). However, only complexes in 30 to 1 and 80 to 1 antigen excess were used as stimuli, and these complexes have been shown in the present experiments to be relatively ineffective in stimulating monocyte pyrogen production. In another *in vitro* model, Chusid and Atkins (60) incubated leukocytes from rabbits hypersensitive to penicillin-protein conjugates with antigen and antiserum and noted endogenous pyrogen production. However, since the blood preparation was undoubtedly a mixture of PMNs and monocytes, the specific pyrogen-producing cell in this system is not known. In the present experiments it is extremely unlikely that contaminating numbers of PMNs are responsible for the observed responses. Less than 1% PMNs were counted in the differential smears of the cell preparations; therefore, no more than 2×10^5 PMNs were present in any flask. Atkins and Bodel (1) reported that any number of PMNs below 5×10^6 produced negligible amounts of pyrogen in response to tuberculin. Contaminating granulocytes, therefore, could

not account for the pyrogen production by incubated monocyte preparations. Lymphocytes are also probably not responsible for pyrogen production in these experiments, since cultures of the non-adherent cells from monocyte-lymphocyte preparations with added complexes did not result in endogenous pyrogen production (Bodel, unpublished observations). In all other systems tested, lymphocytes have failed to produce pyrogen (31). Lymphocytes present in tissue culture flasks may play a role in the observed endogenous pyrogen production. Some lymphocytes were doubtless present, even after non-adherent cells were removed, since the monolayers were not washed in any way. And lymphocytes have well-described receptors for antigen-antibody complexes (45), and exert numerous effects on macrophages (83). It is possible, therefore, that the complexes may act on the lymphocytes, which in turn stimulate the monocytes to release pyrogen.

The observed febrile responses to supernatants from cultures of monocytes plus complexes were clearly due to pyrogen produced in the incubated cells. Complexes incubated without cells in tissue culture medium did not produce significant fevers when injected into test animals in amounts equal to or greater than those present in flasks containing both cells and complexes. Thus, the complexes themselves in those small concentrations were non-pyrogenic in rabbits. Further evidence for this conclusion that the complexes were not acting directly on the cells of the test animal is found in the shape of the fever curve

which looks indistinguishable from a typical endogenous pyrogen mediated response.

The results also demonstrated that, in the case of soluble complexes formed at 10 times antigen excess, the amount of pyrogen released by the cells was directly related to the concentration of complexes added to the cells. This could indicate that a single cell produces more pyrogen when it experiences more interactions with complexes. On the other hand, a minimum number of cell interactions with complexes may be required to stimulate any cell to respond with pyrogen production. When the number of complexes present in the medium is increased, more cells may achieve interactions with the minimum numbers of complexes. It is not known whether stimulation of cells to produce pyrogen is a simple 'off-on' phenomenon, with, in this case, increasing numbers of cells to the active state. One would suspect that if the complexes were evenly distributed throughout the culture medium in the flask, cells would be fairly uniformly exposed to equivalent numbers of complexes. However, if an irreversible attachment to the cell surface or interiorization of the complexes is a prerequisite to stimulation, then the metabolic state of individual cells may dictate which ones are activated more rapidly, consuming or fixing complexes which are then no longer available to other cells which have not as rapidly interacted with enough complexes for activation. Addition of more complexes to the system would then allow a greater number of cells to

be activated.

Some results suggest that the cell preparations do not require continuous stimulation by the complexes for endogenous pyrogen production to continue. In a few experiments, the monocytes were stimulated with complexes, and after 18 hours of incubation, the supernatant fluid was removed from the flask and replaced with fresh tissue culture medium. Reincabation of these cells allowed continued production of significant amounts of pyrogen even in the absence of the complexes. This finding may reflect a fundamental irreversibility to the activated state. Once activated, the cell may be essentially independent of the amount of stimulating substance present. It has been shown in other systems that activation of PMNs completely precedes any pyrogen production (82).

Comparison of the data for cell preparations stimulated with 10 times antigen excess complexes and 50 times antigen excess complexes suggests that qualitative differences in the structure of the complexes may play a role in determining their potency as stimulators of pyrogen production. Arend and Mannik have shown that complexes formed in vivo (75) and in vitro (74) are not uniform in size or molecular antibody-antigen ratio. Using human serum albumin (HSA) and rabbit antibodies to HSA, they showed that soluble complexes formed in a mixture of 5 times antigen excess contained free antibody (20% of total protein), complexes of antibody to antigen of molar ratios of 2:1 (34%), and

complexes of larger size comprised of more than 1 antigen and more than 2 antibodies (46%) (75). A mixture of 20-times antigen excess complexes contained only 32% large complexes and more (48%) of the small complexes. In greater antigen excess there are lesser amounts of large complexes and increased amounts of small complexes, and the same in probably true in our system.

It is clear from our data that the complexes prepared at 50 times antigen excess are essentially ineffective as stimuli of monocyte pyrogen production, while complexes formed at 10 times antigen excess, or 10 times antibody excess are effective. Larger complexes, therefore, may be the active macromolecular moiety in our system, although this is not proven. There may be alternative explanations for our findings. Equal volumes of the various complex mixtures were added to our preparations, in order to eliminate effects of varying the concentration of the culture medium. Therefore equal volumes of complexes of varying antigen antibody proportions were compared. Therefore, 5 fold less total antibody was present in the 50-times antigen excess preparation than in the 10 times antigen excess preparation (high dose). Since, as noted above, there is some dose relationship for activation of the cells, the observed negative responses at 50 times antigen excess may be due to inadequate total dose of complexes, when measured by the antibody moiety, rather than qualitative differences in complex structure per se. However, when complexes were

prepared at 20-times antigen excess (data not included in results because only 8 values were obtained), the average fever response was only 0.25°C, corresponding to a pyrogen dose of less than 1.0 units. These 20-times antigen excess complexes did contain amounts of antibody equal to that present in the low dose 10-times antigen excess complexes, which were shown to stimulate significant amounts of pyrogen production (see Fig. 7). So while the ineffectiveness of the 50 times antigen excess complexes may be a result of dose effects, it appears that qualitative aspects of the complexes also play a role.

The 50 times antigen excess complexes may be poor stimuli as a result of the ratio of globulins present, since in this system the antigen is itself an immunoglobulin. Although commercially prepared BGG probably contains mostly denatured protein, some intact BGG is probably also present. If the mechanism by which complexes stimulate monocytes is a specific interaction between the Fc region of antibody in complexes and a specific immunoglobulin receptor on the cell surface, then free immunoglobulin may competitively inhibit the interaction, as is observed in other cell-complex interactions. Free BGG present in the antigen excess mixture, therefore, may exert an inhibitory effect on the complex-mediated stimulation of cells. Arend and Mannik (39) have shown that there is only partial species specificity for inhibition of cell-complex adherence, so the exact effects of free BGG, or even free rabbit antibody, on the

cell-complex interactions in this system are unknown.

Similar mechanisms of competitive binding, and low dose of complexes may be playing a role in the response observed with antibody excess complexes. While they clearly had a positive stimulating effect on cells, in the amounts given, the statistical mean of the pyrogen dose produced is lower than that observed with the same "dose" of 10 times antigen excess complexes (1.9 ± .70 pyrogen units compared to 2.3 ± .50 units for antigen excess), although there is some probability that the statistical means might be in the same range. Cells may have, in fact, received a lower dose of antibody excess complexes. When preparing complexes in vitro it was observed that the gross size of the precipitate pellet was much larger in the antibody excess tube as compared to the antigen excess precipitate which appeared minuscule. So the total dose of complexes present in a given volume of the antibody excess supernate may well have been considerably lower than that present in the antigen excess supernate. Additionally, if the complex-cell interaction is inhibited by free antibody, then free rabbit antibodies present in antibody excess mixtures may have had an inhibitory action of the stimulating effect of the complexes which were present. Considered together, this might indicate that if an active fraction of complexes could be separated from the total complex mixture, complexes formed in antibody excess might, in fact, be a more potent stimulus for pyrogen production than complexes formed at antigen excess.

That is, a range of larger complexes may be the active molecular species. By preparing complexes in antigen excess, one may be decreasing the total dose of the active molecular fraction by forming increased amounts of small complexes of antibody to antigen ratio of 2:1 or less. These small complexes may render the small amount of large complexes ineffective by directly inhibiting the action of the larger complexes present, just as does the free BCG. Phillips-Quagliata (46) showed that complexes with monovalent antigens not only fail to attach to cells, but also markedly inhibit the attachment of complexes with polyvalent antigen. Thus, small complexes present in large amounts in far antigen excess may represent complexes of functionally monovalent antigen which are both non-stimulatory themselves, and inhibitory to the attachment of adequate numbers of the active, larger complexes.

Precipitated complexes did not stimulate monocytes to produce pyrogen in this system, even though they contain relatively large complexes. At equivalence, the calculated antibody to antigen ratio for the precipitated complexes is approximately 3:1, antibody to antigen; in antibody excess, complexes of this size or larger are probably present. In a similar system, precipitated complexes are readily interiorized by macrophages, localized in phagolysozomes, and subsequently degraded (38). It may be argued that perhaps the cells did not phagocytose the precipitates. It was not demonstrated that such phagocytosis in fact took place. How-

ever, the cells were clearly capable of phagocytosis of staph particles and capable of pyrogen production while in the presence of the precipitates, and mononuclear cells are known to phagocytize a wide variety of particulate substances including cellular debris, without a requirement for opsenizing antibodies. And the microscopic appearance of the cells in the presence of precipitates suggested widespread and extreme cell activation, the cells appearing grossly enlarged, granular and clumped into large aggregates. This corresponded in appearance to that observed in flasks in which phagocytosis of heat killed staphylococcal particles had occurred. So while not proven, it is suggested that the cells phagocytosed the complex precipitates, but were not stimulated to produce the pyrogen.

Consequently, although phagocytosis in our system was not proven, it is likely that the production of pyrogen may not be simply a nonspecific concomitant of phagocytosis of particles. Phagocytic cells are known pyrogen-producing cells (1,25,29) and, in general, greater amounts of pyrogen are produced in response to larger amounts of phagocytic stimulus (1). There are numerous kinds of particles which are readily phagocytosed by such cells (34). However, many soluble substances are adequate stimuli also, such as etiocholanolone (20), tuberculin (1) and endotoxin (Bodel, unpublished observations). And since the stimulus for pyrogen production in the presence of immune complexes is presumably not phagocytosis per se, a more specific complex-

cell interaction must be postulated to account for the observed stimulation by the complexes in solution.

The most likely mechanism by which complexes stimulate the cells to pyrogen production may be through specific attachment to the immunoglobulin receptors on the cell surface. In the case of precipitates, such interactions may be infrequent. It is well known that the process of formation of antigen-antibody complex is a two step process: first attachment of the antibodies by the Fab region attaching site to numerous exposed antigenic determinants on the antigen molecule. The process of precipitation per se is due to noncovalent intermolecular interactions between numerous such antibodies on one or more antigens in such a way that water is excluded from the complex, and it forms an insoluble precipitate (84). So, in precipitate form, the specific region of the Fc fragment required for interaction with cells may be bonded noncovalently to other antibodies and thus unavailable for attachment to the specific cell receptor altogether, and/or the combining site may have too much simple steric interference from neighboring molecules. This theory, then, would suggest that precipitates are probably phagocytized non-specifically as particles, whereas soluble complexes interact first with numerous adjacent cell surface receptors, thereby stimulating activation of the cell processes, including pyrogen production.

Precipitates may have failed to initiate pyrogen production through alterations in the availability of complement.

Precipitated complexes, unlike soluble ones, were washed, removing other rabbit serum factors,and one might question whether absence of complement was the reason the precipitates seemed to elicit no pyrogen response. However, rabbit antibody fixes human complement (85), and the tissue culture medium contained 15% fresh human serum, presumably an adequate source of complement.

Studies of the factors required for activation of monocytes by soluble complexes were limited. Several experiments, however, showed clearly that heating both rabbit and human sera prevented all activation (Bodel, unpublished observations). This data suggests the importance of complement to the reaction.

While much literature data on the interaction of complexes with cells, both in vivo and in vitro, has been presented on the role of complement in such reactions, the exact role complement plays in this system remains to be defined. Complement factors may stimulate pyrogen production quite independently of the actual cell-complex interaction. This seems unlikely, since complement fixing precipitates should, then, activate the cells. On the other hand, cells may be activated by a theoretical cell-complex interaction quite independently of a concomitant complement consumption reaction. It has been proposed that in vivo uptake of large complexes, which fix complement most readily, is a phenomenon quite separate from their observed in vitro complement fixing abilities (75). It has been also suggested

by Mickenberg et al (73) that the pyrogen producing ability of large complexes formed in antibody excess is a complement-independent phenomenon, since cobra venom factor-treated rabbits retain febrile reactivity to such complexes, unlike smaller complexes which are formed in antigen excess. However, much data supports the view that complement does play a role in the pathogenesis of immune fever. For example, Mickenberg showed convincingly that complement depletion eliminated febrile responsiveness to antigen excess complexes (73).

One hypothesis which might explain the apparently conflicting results is that complement independent attachment of soluble complexes to cells in this system may indeed take place concomitantly with a probably complement dependent mechanism of pyrogen production. According to this theory, the phenomenon of stimulation of pyrogen production may be a separate one from that of attachment and subsequent interiorization by the cell.

It is apparent from our data and from that of others (73), that there is a finite range of size (lattice size) of antigen-antibody complexes which activate mononuclear cells to produce pyrogen, or cause the release of enzymes polymorphonuclear leukocytes (90). However, Arend and Mannik have shown that clearance of antigen-antibody complexes of all sizes from the circulation of animals is virtually complete, 99 percent being removed by the liver (75). Clinical evidence suggests that the immunopathologic

activity of complexes is not primarily due to presence of complexes in the circulation, but rather to abnormal localization of complexes in sites from which they are not rapidly cleared, e.g. in the kidney (86), in the walls of blood vessels (87), in the cerebrospinal fluid space (88). This suggests that uptake of complexes from the circulation by cells of the reticuloendothelial system may not be the mechanism of pyrogen production. It may be that separate, probably complement dependent, interactions take place between cells and soluble complexes of large size (>11S sedimentation coefficient) resulting in activation of mononuclear cells, with the production pyrogen as one result.

When Mickenberg et al showed that antigen excess complexes of human serum albumin (HSA) and rabbit anti-HSA, lost their fever producing capability in decomplemented rabbits, while antibody excess complexes retained fever producing ability in such animals, they concluded that "large" complexes formed in antibody excess produced fever independently of complement, that the in vitro complement fixing ability of the large complexes only "paralelled" their fever producing activity. They concluded that "small" complexes in the antigen excess mixture were probably not representative of the complexes which form when antigen is injected into an immune animal, and could be disregarded as being responsible for any fever producing activity produced by injection of the antigen. However, Arend and Mannik have shown that large complexes are, in fact, present in mixtures of 10

(and greater) times antigen excess. Perhaps what happened in Mickenberg's (73) experiments is that complexes of sufficient size, but at the small end of the range of active complexes present in the antigen excess mixture produced fever by a complement dependent mechanism. Repeated injections of complexes produced increasing consumption of complement components to the point where the smaller, relatively inefficient complement fixing complexes could no longer activate enough complement sufficiently rapdily to produce activation of cells. Similar (partial) depletion of complement, by treatment with cobra venom factor, rendered the complexes again incapable of initiating activation of the host cells to make pyrogen. However, when large complexes, at the larger end of the range of active complexes, were injected, their more efficient complement fixing ability allowed them to initiate a (complement dependent) response even in the face of (partial) complement depletion. Indeed, further reduction in complement was observed when the antibody excess complexes were injected. That the large complexes consumed less complement in vivo, in a normal animal, than did antigen excess complexes, can be accounted for on the basis of their much shorter life span in the circulation. Clearance of complexes by the RES may be what stopped their activity in stimulating cells to produce pyrogen, rather than what produced the pyrogen.

Obviously, data is required to determine exactly what

in vitro mechanisms are in fact operating to lead to cell stimulation. The theory presented herein accounts for observed phenomena in Mickenberg's *in vivo* system, and is consistent with the observed data for complex-cell interactions as well as the present finding that phagocytosis of complexes *per se* does not result in pyrogen production. Coincidentally it offers a possible theory of febrile complex tolerance, i.e. complement depletion, which can account for complement dependent cell activation to pyrogen production in the face of complement independent mechanisms observed in uptake of complexes by macrophages *in vitro* and clearance of complexes *in vivo*.

Similar mechanisms of complement dependent cell activation without phagocytosis have been proposed in other systems. Miller *et al* (89) have observed complement mediated release of complexes from lymphocyte surfaces, involving the properdin pathway of complement activation. The authors propose that antigen-antibody induced activation of the normal complement sequence leads to attachment of an antigen-antibody-complement complex to the cell surface. However, subsequent activation of the alternate pathway produces intermediates which then cause the release of the complexes, thereby mediating a possible *transient* association of antigen-antibody complexes with the cells, possibly resulting in some form of cell activation without concomitant phagocytosis. Certainly such a mechanism is an attractive one for interpretation of the complex induced cell activation leading to production of

pyrogen.

Many aspects of immune fever, both experimental and clinical, remain unclear. What is the exact nature of the complex-cell interaction? Is pyrogen production a necessary concomitant of attachment and phagocytosis of complexes by cells of the RES, or can uptake and clearance of complexes occur without pyrogen production? What other cells are capable of being activated by complexes? Which cells are the active ones in various hypersensitivity disease states? Are other mechanisms of cell activation involved? Does complement play a role in attachment? interiorization? cell activation? What mechanisms operate *in vivo* to result in defective clearance of complexes and localization of complexes in sites where they mediate inflammation and injury? Exactly what kinds of complexes are active stimulators of cells? What antibody class and subclass specificity exists for fever production? What kinds of naturally occurring exogenous and endogenous antigens are responsible for observed clinical hypersensitivity states in which fever is a part of the symptom complex?

Hopefully, further studies using our *in vitro* system may help to answer some of these questions. When various molecular species present in a reaction mixture of complexes are separated, the effectiveness of large and small species can be directly evaluated. Further experiments with decomplemented serum need to be carried out to determine the exact complement requirements of the pyrogen production

phenomenon. Use of different antigens may help elucidate mechanisms of fever production in clinical states associated with antigen-antibody complexes involving host antigens such as DNA, and glomerular basement membrane. And much remains to be done to determine the relations between the mechanisms of immune fever production and those involved in the pathogenesis of immune complex disease.

SUMMARY

Human peripheral blood monocytes were stimulated *in vitro* by soluble antigen-antibody complexes to produce endogenous pyrogen. A dose-dependent relationship was observed between pyrogen production and the amount of immune complexes present in the medium surrounding the cells. Qualitative differences in the size of complexes were inferred to play a role in determining the potency of complexes as cell stimulators. Precipitated antigen-antibody complexes were inactive as stimuli for pyrogen production. Theories of *in vivo* immune fever production are proposed, on the basis of the present findings.

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